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A Comprehensive Handbook

Edited by
K. Drauz and H. Waldmann

Volume 1

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A Comprehensive Handbook

Edited by
Karlheinz Drauz and Herbert Waldmann

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Foreword

That biological systems are masterful chemists is a fact long appreciated by those who study how living things build complexity from simple compounds in the environment. Enzymes catalyze the interconversion of vast numbers of chemical species, providing materials and energy to fuel cell survival and growth. Enzymes build the intricate natural products, which, for their potential utility in treating disease, pose almost unlimited new challenges for ambitious synthetic chemists. But, unlike most industrial chemical processes, Nature's catalysts generate few waste products and effect their transformations under mild conditions–in water, at room temperature and atmospheric pressure. Biocatalysts are models of energy-efficient, environmentally-conscious chemistry and will play a prominent role in the 21st century's chemicals industry.

The world of biocatalysis has undergone significant change in the eight years since the first edition of this handbook appeared. Most of the news is good, with enzymes showing up in many more organic syntheses and a number of important new industrial processes coming on line. Apart from continuing clever insights into how to integrate biocatalysis into synthetic chemistry, several forces are accelerating a move to biocatalytic processes. In the first place, the search for better, enantiomerically pure drugs has forced many chemists to turn to enzymes for assistance in their preparation. Ever increasing demands for environmentally acceptable processes push in the same direction. At the same time, rapidly-developing technologies for making better catalysts through genetic enginering and for discovering new catalysts are are offering new process opportunities which in the past were either not economical or not even conceivable. A plethora of new catalysts to choose from, as well as a high probability that a catalyst can be further improved during the process design and engineering phases, means that we can respond rapidly to new synthetic needs with biocatalytic solutions.

The organization of these volumes into specific technologies and transformations provides a comprehensive coverage of practical biocatalysis that no other single source provides. The work of experts in each of the fields, the individual chapters review vast relevant literature and synthesize it in order to present key concepts and many illustrative examples. This coverage should give organic chemists immediate access to the wealth of experience that has accumulated in the biocatalysis world and allow them to identify the most promising ways to use biocatalysts in their own

syntheses. Biocatalysts should feature prominently in the repertoire of synthetic chemistry, and this handbook deserves a prominent place in the modern chemist's library.

Pasadena, January, 2002 *Frances Arnold*

Preface

Nearly eight years have passed since we the First Edition of „Enzyme Catalysis in Organic Synthesis" was issued but much of what we had written in its preface then still applies today. The application of biocatalysis in organic synthesis is a powerful technique. It has grown steadily and today this field is well-established in both academia and industry. With increasing application and acceptance the need for a comprehensive and up to date overview of the state of the art has grown. In addition numerous colleagues have approached us and asked for an update of "the Handbook".

In response to these demands and in recognition of the new and groundbreaking strides taken since the first half of the nineties the Second Edition which is now in the hand of the reader was prepared. In comparing it with the First edition one discovers that we have not changed the overall arrangement in the volumes. Therefore we continue to have a part that addresses general principles (Chapters 1–10) and another one which summarizes the application of enzymes in organic synthesis according to reaction type (Chapters 11–20). This arrangement was very well received by the readers before and we hope that it will be for the Second Edition as well.

However, the entire text was streamlined and in many cases regrouped to ensure for a better presentation. Also a few chapters which in the long run turned out to be less relevant to organic synthesis were not included again. In contrast other aspects were now integrated and attention was given to techniques of enzyme evolution, bioinformatics and enzymatic reactions in low-water media, areas that have developed with great pace and that we believe to be of major importance in the time to come.

We hope that the Second Edition of the "Handbook" will be a plentiful source of information just as valuable as the First Edition was eight years ago.

Dortmund and Hanau, February 2002 *Karlheinz Drauz, Herbert Waldmann*

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1
Introduction

Maria-Regina Kula

1.1
Enzymes as Catalysts

Enzymes are the catalysts evolved in nature to achieve the speed and coordination of a multitude of chemical reaction necessary to develop and maintain life. Chemical reactions are far too slow to be effective under the conditions prevalent in normal living systems – aqueous environments with neutral pH values and temperatures between 20 and 40 °C. Even catalysts developed in the chemical industry fall short; enzymes in comparison achieve up to 10^7 – fold faster reaction rates. Mankind has utilized enzymes empirically since ancient times for the conservation or production of food, e.g. in cheese making or brewing. A historical background is given in Table 1-1. The catalytic properties of enzymes were recognized long before their chemical nature was known. We still use acceleration of reaction rate to search for unknown enzymes as well as to measure and quantify enzyme activity.

As catalysts – true to the definition familiar in chemistry – enzymes alter the rate at which a thermodynamic equilibrium is reached, but do not change that equilibrium. This implies that enzymes work reversibly. The acceleration in reaction rate is achieved by lowering the activation energy of the overall process as shown schematically in Fig. 1-1.

Enzymes bind their substrates by multiple non-covalent interactions on a specific surface. This way, a micro-heterogenization occurs and the local concentration of substrates is increased relative to the bulk solution. In addition, the chemical potential of specific groups may be drastically changed temporarily compared to aqueous solutions by the exclusion of water in the reactive site upon binding of substrate. Both aspects contribute to the observed phenomenon of high acceleration in reaction rate; some examples are presented in Table 1-2. Enzymes often bind the substrate in the transition state better than in the ground state, which lowers the activation energy.

Since the pioneering work of Buchner (1897), it has been known that enzymes do not require the environment of a living cell to be active. This opened the way to many applications in food technology, in the production of leather, textiles and paper, in

Table 1-1. Brief history of enzymes and their applications.

B C	Chymosin from the stomach of young cattle, sheeps and goats was used for cheese production in many ancient cultures for approximately 7000 years.	
1783	Hydrolysis of meat by gastric juice demonstrated.	Spallazani
1814	Starch degradation and sugar production by malted barley observed.	Kirchhoff
1833	The active principle of malt is called diastase and its application to industrial art described.	Payen and Persoz
1846	Invertase activity observed.	Dubonfout
1867	The term *enzymes* is coined to describe catalytic activity not bound to living cells (unorganised ferments). The name is extended later also to intracellular catalysts (organised ferments as defined by Pasteur).	Kühne
1893	Definition of a catalyst including enzymes is given.	Ostwald
1894	Enzyme stereospecificity anticipated.	E. Fischer
1894	"Taka diastase" produced commercially with *Aspergillus oryzae* by surface culture	Takamine
1897	The conversion of glucose to ethanol demonstrated by a cell free extract from yeast.	Buchner
1906	Preparative separation of L-leucine from the racemate carried out by hydrolysis of the propyl ester with liver extracts.	Warburg
1908	Synthesis of optically active cyanohydrins described, using D-oxynirerilase from almonds as catalyst.	Rosenberg
1908	Application of pancratic enzymes in the leather industry for the bating of hides.	Röhm
1911–1913	Glucoside synthesis in the presence of high concentration of ethanol or acetone described.	Bourquelot, Bridel and Verdon
1913–1915	Application of pancreatic enzymes to clean laundry introduced, first commercial product sold to the public: Burnus.	Röhm
1916	Immobilization of invertase on charcoal demonstrated with retention of activity.	Nelson and Griffin
1926	Urease from Jack beans crystallized.	Sumner
1936	Enzymatic ester synthesis improved using pancreatic lipase in the presence of benzene.	Sym
1953	The first primary sequence of a protein (Insulin) established, proving the chemical identity of proteins.	Sanger
1960	Cultivation of *Bacillus licheniformis* in submerged culture started for protease production on a large scale.	NOVO
after 1980	Application of genetic engineering techniques to improve enzyme production and to alter enzyme properties by protein engineering and evolutionary design.	many

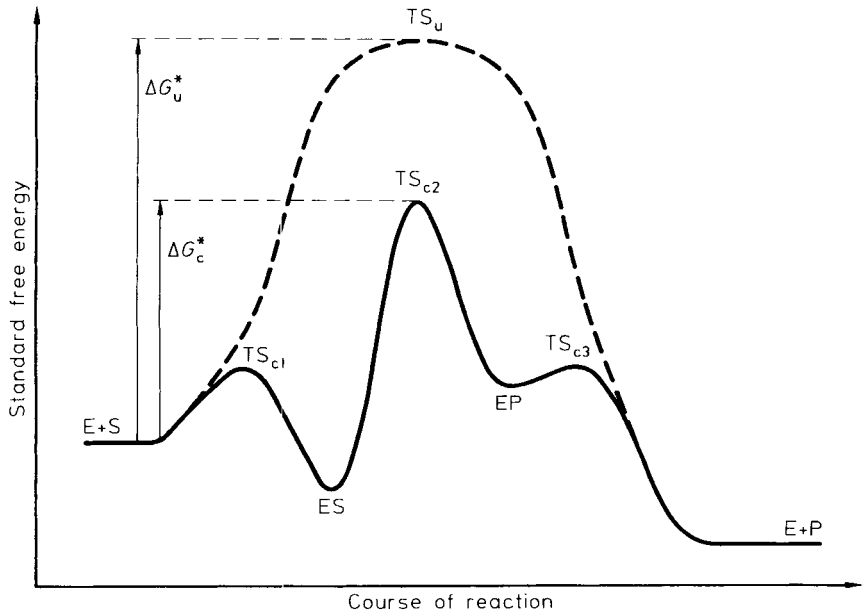


Figure 1-1. Free-energy profile for the course of an enzyme-catalyzed reaction involving the formation of enzyme-substrate (ES) and enzyme-product (EP) complexes. The reaction pathway goes through the transition states TS_{c1}, TS_{c2}, and TS_{c3} with standard free energy of activation ΔGc. The rate limiting step would be the conversion of ES into EP. The schematic profile for the uncatalyzed reaction is shown as the dashed line. The catalytic effect is due to the lowering of the standard free energy of activation from ΔGu to ΔGc and is not governed by the difference in free energy between S and P.

diagnostics and food analysis and, last but not least, in the production of chemicals by biotransformations.

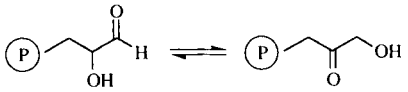
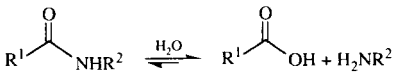
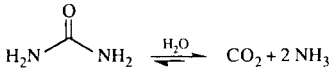
One or more of the following reasons could make enzymes the catalysts of choice:

- a highly selective operation in complex mictures,
- stereo- and regiospecificity of conversions,
- absence of side reactions leading to simpler separation processes and higher yields, or
- savings in energy and waste treatment cost owing to mild reaction conditions.

Enzymes have limitations, as does any other highly specialized catalyst. Most notable is one consequence arising from the selectivity of enzymes with regard to the substrates bound and the type of reaction catalyzed. The price for such selectivity is that it may be difficult to satisfy the requirement for many special enzymes to cover the diversity of chemical reactions desired in organic chemistry. The enzyme needed in a specific case may not be readily available. However, there are new enzymes discovered all the time and an increasing number can be obtained commercially.

Other limitations with regard to reaction conditions, pH and temperature tol-

Table 1-2. Relative rates of enzyme catalyzed and non-catalyzed reactions under conditions optimal for enzyme reaction.

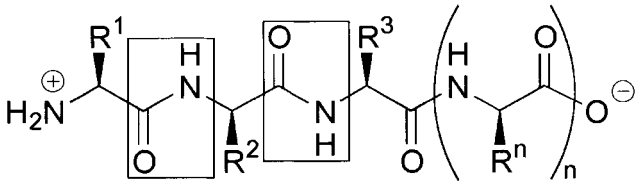
Enzyme	Reaction	Ratio[a]
Triose phosphate Isomerase		3×10^{8}–10^{9}
Serine protease		10^{5}–10^{10}
Urease		10^{14}
Hexokinase[b] glucose + ATP → glucose 6 Ⓟ + ADP		$> 8\times10^{10}$ M
Alcohol[b] dehydrogenase ethanol + $NAD^{\oplus}$ → acetaldehyde + NADH + $H^{\oplus}$		$> 2\times10^{10}$ M

a ratio of enzyme catalyzed to non-catalyzed rate
b bimolecular reactions
Ⓟ = Phosphate

erated by enzymes are to some extent predictable by their chemical nature. In this Introduction, general aspects of enzyme structure, function and nomenclature will be discussed to guide the reader with little biochemical background into the field of enzyme application in organic chemistry.

1.2 Enzyme Structure and Function

All enzymes are proteins, with the exception of the recently discovered ribozymes. Ribozymes are special ribonucleic acids performing catalytic functions in the processing of RNA which will not be considered here. Proteins are polar macromolecules with molecular mass in the range 10^{4}–10^{6}. They are linear polymers, defined by the sequence of amino acids, which are linked by peptide bonds:



The individual properties of a protein depend on the chemical nature of the side chains depicted as R in Scheme 1-1. In protein biosynthesis, 20 amino acids are

Table 1-3. Amino acids for protein biosynthesis.

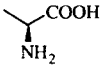
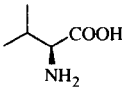
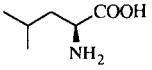
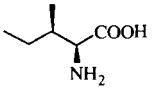
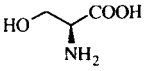
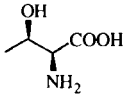
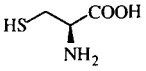
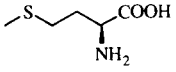
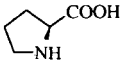
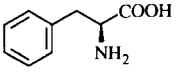
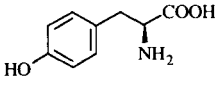
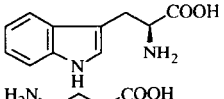
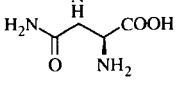
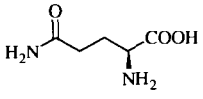
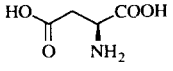
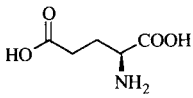
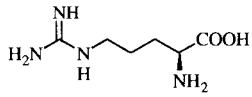
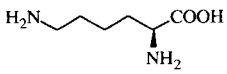
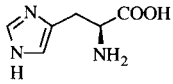
Name	Symbol	Structure	pK_a of ionizing side chain[a]
Glycine	Gly (G)		
Alanine	Ala (A)		
Valine	Val (V)		
Leucine	Leu (L)		
Isoleucine	Ile (I)		
Serine	Ser (S)		
Threonine	Thr (T)		
Cysteine	Cys (C)		9.1–9.5
Methionine	Met (M)		
Proline	Pro (P)		
Phenylalanine	Phe (F)		
Tyrosine	Tyr (Y)		9.7
Tryptophane	Trp (W)		
Asparagine	Asn (N)		
Glutamine	Gln (Q)		
Aspartate	Asp (D)		4.5

Table 1-3. (cont.).

Name	Symbol	Structure	pK_a of ionizing side chain[a]
Glutamate	Glu (E)		4.6
Arginine	Arg (R)		~12.0
Lysine	Lys (K)		10.4
Histidine	His (H)		6.2
α-amino group			6.8–7.9
α-carboxyl group			3.5–4.3

a The pK_a values depend on temperature, ionic strength and, especially on the microenvirronment of the ionizable group

condensed according to information coded in the corresponding genes. The coded amino acids are summarized in Table 1-3. Some modified amino acids, for example 4-hydroxyproline, 5-hydroxylysine, γ-carboxyglutamate, O-phosphorylated serine, or N-glycosylated asparagine are also found in proteins, usually in minor amounts, resulting from post-translational modifications. These modifications are important for the structure and function or the regulation of the activity of certain proteins. For 20 building blocks and a random sequence, the number of possible variations in the primary structure is 20^n; for a protein of average size of 33 000 Da ≙ 300 amino acids, 10^{390} possibilities exist. The number is far beyond our perception, the known cosmic space is not large enough to accommodate a single copy of each variant. To generate a specific surface as part of the active center of an enzyme, the protein chain has to fold. From the observed length and angle of the C=O and C–N bonds in peptides it can be deduced that the peptide bond possesses partial double bond character, resulting in a planar arrangement as shown schematically in Fig. 1-2. Movement of the planes against each other occurs around the α-carbon, which serves as a joint (Fig. 1-3). Rotation around the C–C and C–N bond is restricted because of steric influences of the side chain R. By this feature of the peptide bond, two structural arrangements of a polypeptide become energetically favored: the α-helix and the β-pleated sheet, which are further stabilized by hydrogen bonds between the peptide backbone (Fig. 1-4). Helices and pleated sheets are commonly found in proteins; these secondary structure elements are linked by β-turns or loops to build a domain or a subunit. This level of organization is called the tertiary structure of proteins, while the assembly of subunits into homo- or hetero-oligomers or multi-component systems is called quaternary structure. The hierarchy of structures is depicted in Fig. 1-5.

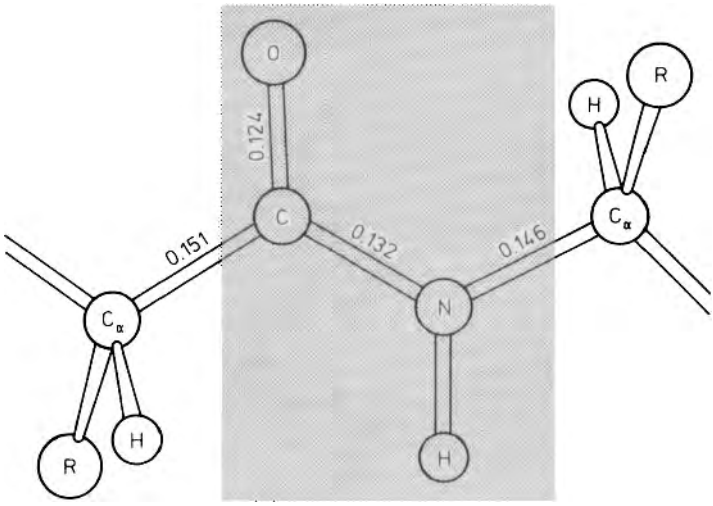


Figure 1-2. Special features of the peptide bond. Dimensions are given in Å and represent average values from X-ray analyses. The peptide group has a rigid and planar structure.

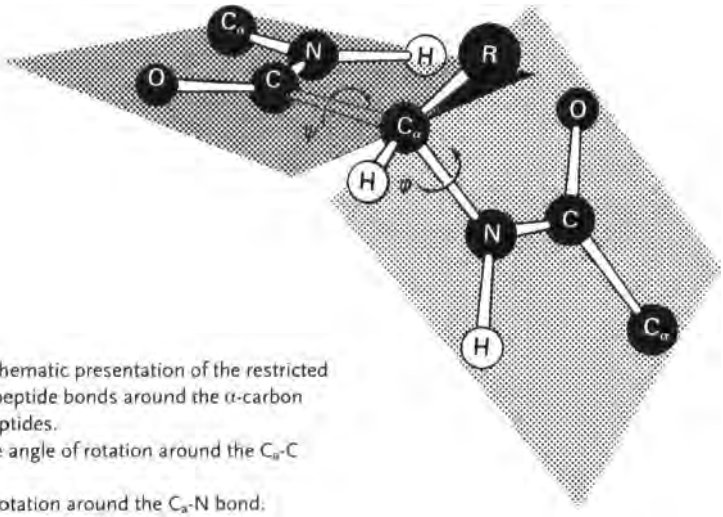
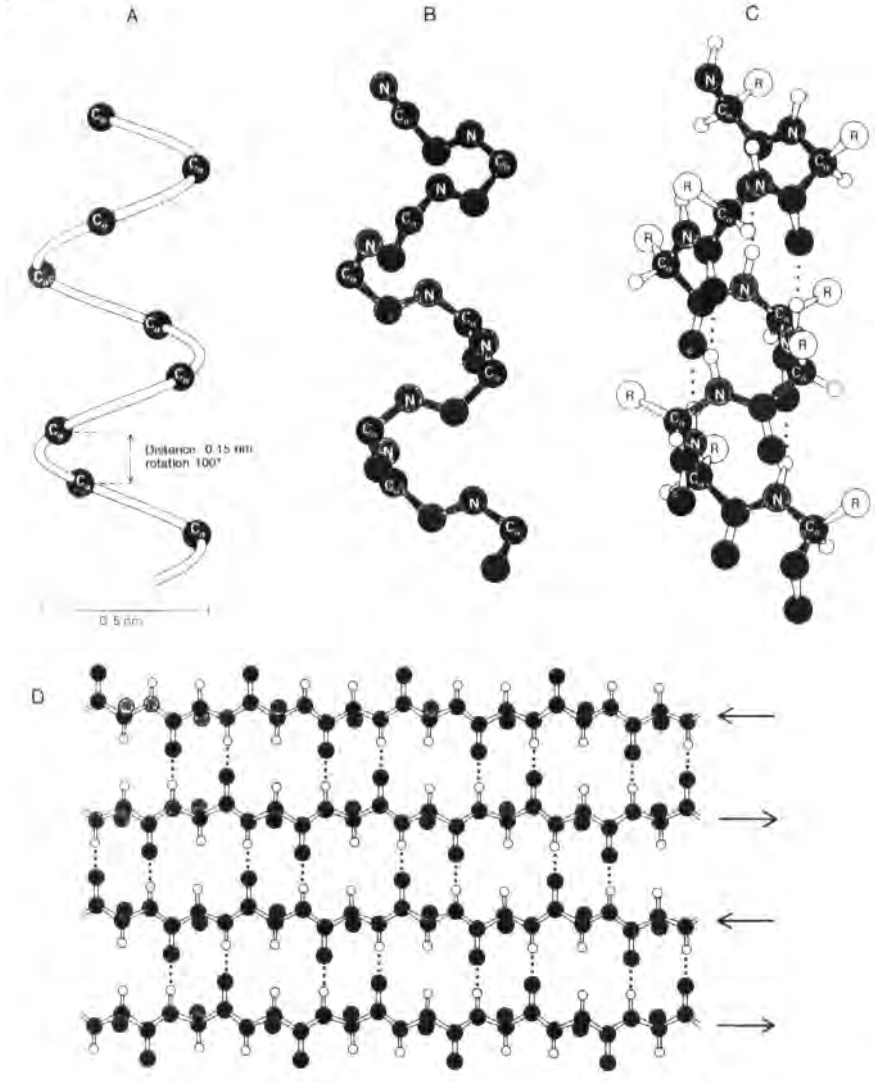


Figure 1-3. Schematic presentation of the restricted orientation of peptide bonds around the α-carbon atom in polypeptides.
ψ describes the angle of rotation around the C_α-C bond,
θ the angle of rotation around the C_α-N bond.

The mechanism determining the folding of a given protein is presently the topic of intense research. For this discussion, it is sufficient to state that there exists a unique tertiary/quaternary structure for each native protein chain, determined as an energy minimum in aqueous solution. The information to reach this structure is thought to be encoded in the primary sequence in a way not yet understood completely. The



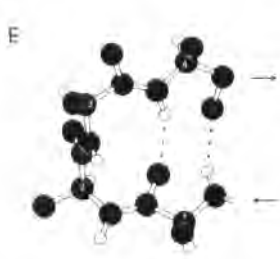


Figure 1-4. Illustrations of a helices (A, B, C), β-pleated sheets (D) and β-turns (E), the common elements of secondary structure in proteins. From a right handed α-helix is shown in A: only the α-carbon atoms of the peptide backbone; B: α-carbon (C_a), nitrogen (N) and the carbonyl carbon (C) of the peptide backbone. C: complete helix with stabilizing hydrogen bonds between NH and CO groups, R stands for the different side chains of amino acids. An antiparallel β-pleated sheet is depicted in D. Adjacent strands may run in opposite directions (as shown) or alternatively in parallel directions. Hydrogen bonds between adjacent strands stabilize the structure. The side chains R are above or below the plane. The structure of a β-turn is shown in E. The carbonyl group of amino acids 1 is hydrogen bonded to the NH group of amino acid 4 resulting in a hairpin turn.

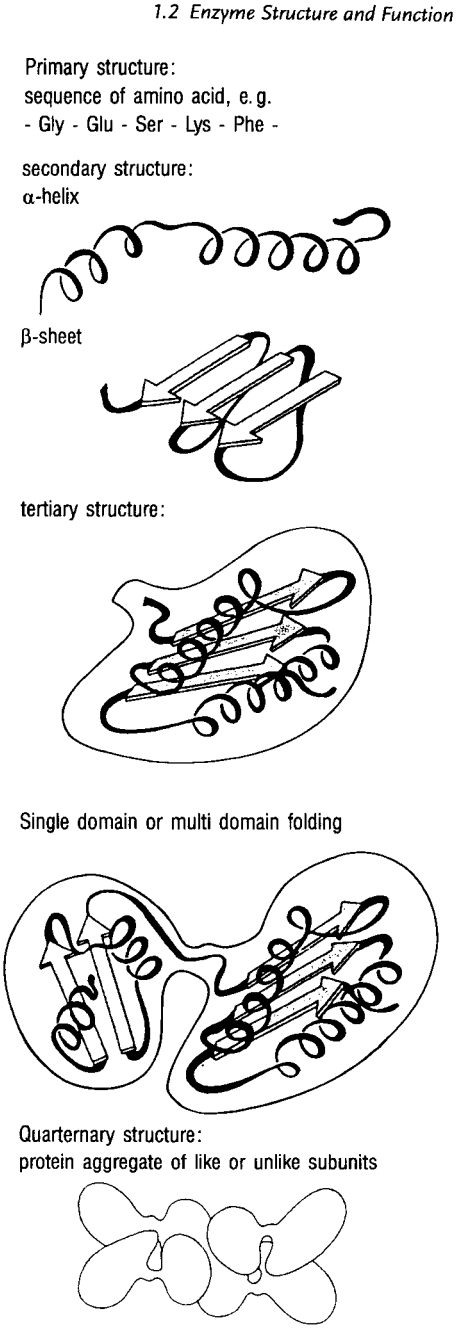


Figure 1-5. Hierarchy of protein structures. The three-dimensional structure of an enzyme is the result of different levels of folding and interactions of protein chains proceeding in ordered fashion from the primary structure after protein biosynthesis.

folded structure of a protein is stabilized by a network of non-covalent interactions, most notably hydrogen bonds, hydrophobic and van der Waals interactions, and ionic bonds. In the folding process, hydrophobic side chains of amino acids are

preferentially oriented towards the interior of the molecule, thereby diminishing the surface area in contact with water and minimizing the free energy. Polar groups are preferentially oriented towards the surface interacting with water. In the compact inner core of a protein, water is virtually excluded or present as single H_2O molecules (!) in defined places. The folding process generates a unique three-dimensional surface of a protein defined in molecular dimensions by the specific side chains and the polypeptide backbone.

Substrates and their transition states are also bound by multiple noncovalent interactions with such a surface. Since the strength of all these noncovalent bonds is strongly dependent on distance and angle of interaction, a highly selective binding may result. By a three-point attachment even discrimination between two enantiomers is possible. Steric constraints may also contribute to differentiation between similar structures during binding. The specific binding site of enzymes often is found in a cleft on an irregularly shaped surface. Substrate recognition is a dynamic process not only with regard to association and dissociation of the substrate; it may also involve movements of the polypeptide chain in response to the binding. An example of the latter is shown in Fig. 1-6.

Carboxypeptidase A hydrolyzes proteins sequentially starting from the free carboxyl terminus. The enzyme preferentially cleaves hydrophobic amino acids. Already in 1967 the three-dimensional structure had been determined with high resolution by W. Lipscomb and his group. In the meantime much is known about the catalytic mechanism. The essential features are discussed here briefly to improve the understanding of how enzymes work. Two aspects of enzymatic catalysis can be illustrated by this example: 1. Substrate binding may be accompanied by changes in enzyme structure. 2. Substrate binding induces subtle but important shifts in electron distribution in the substrate, making it more susceptible to certain reactions (here hydrolysis).

In Fig. 1-6, the tertiary structure of the free carboxypeptidase A is presented as well as an enzyme-substrate complex with glycyl-tyrosine. Changes in the enzyme structure are most evident by looking at the position of tyrosine-248. The phenolic hydroxyl group of the side chain moves from a position near the surface of the enzyme 12 Å toward the interior. A distance of 12 Å represents about a quarter of the diameter of carboxypeptidase A. Tyrosine-248 then covers the bound glycyltyrosine (Fig. 1-6 B) and the phenolic hydroxyl group is oriented toward the terminal carboxyl group of the substrate. The movement of the tyrosine-248 side chain is possible by rotation of the C – C bond at the β carbon. As a consequence of the rotation, the binding site of carboxypeptidase A is shielded from bulk water. Closer inspection of Fig. 1-6 A and B shows that the guanido group of arginine-145 as well as glutamate-270 also move about 2 Å upon substrate binding. Both residues are involved in the catalytic step.

The second important point is the perturbation of the electron distribution in the substrate by the essential Zn^{2+} and specific side chains in the enzyme. During the binding process the substrate is oriented first by an electrostatic interaction of the carboxylate group with the positively charged arginine-145; in addition, tyrosine-248 forms a hydrogen bond with the amide nitrogen of the terminal peptide bond. The carbonyl group becomes coordinated to the Zn^{2+} displacing water as a ligand.

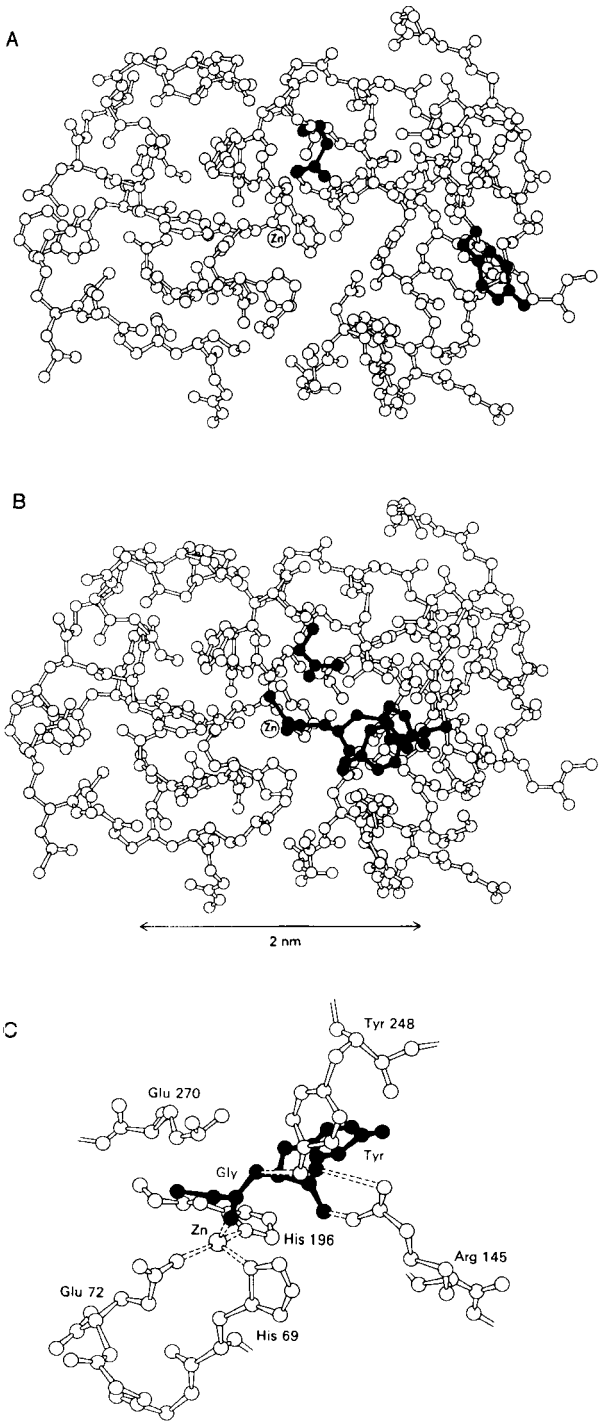


Figure 1-6. The structure of carboxypeptidase A changes dynamically upon substrate binding. (A) Enzyme alone, (B) enzyme complex with glycyl-tyrosine. Tyrosine 248 moves 12 Å after binding of substrate. Hydrolysis results as a concerted action of Zn^{2+}, Glu, Tyr, and Arg side chains towards the carbonyl and nitrogen group in the susceptible peptide bond (C).

The hydrophobic group in the substrate (tyrosine in the example illustrated here) is bound into an unpolar, large cleft by hydrophobic forces replacing at least 4 water molecules upon binding and inducing the movement of tyrosine-248 discussed above. The unpolar lining and the size of the cleft explains the preference of carboxypeptidase A for bulky, hydrophobic side chains of the terminal amino acid. The free amino group of glycyltyrosine is hydrogen bonded through a water molecule to glutamate-270. This bonding of glutamate is thought to slow down dramatically the hydrolysis rate of glycyltyrosine and related dipeptides (and make possible the X-ray analysis of the complex). Such a hydrogen bond is not found in productive enzyme-substrate complexes involving oligopeptides or proteins. The carboxylate group of glutamate-270 is thought to attack the carbon in the carbonyl bond of the substrate leading to a mixed anhydride. The carbonyl bond is already polarized by the Lewis acid Zn^{2+}, the induction of the dipole is favored by the unpolar surrounding of the Zn^{2+} ion and the tetrahedral intermediate is stabilized by the positive charge of nearby arginine-127. The hydrolysis of the peptide bond is completed by transfer of a proton form water to the nitrogen, releasing the C-terminal amino acid.

Substrate binding in a defined manner is a prerequisite for enzyme catalysis. It exposes a chemical compound long enough to a unique chemical potential built into the system, which defines the type of reaction that will proceed, for example, hydrolysis, oxidation/reduction, or C – C bond formation. The mechanism most often is the same as that known from solution chemistry, for example, acid-base catalysis. The close proximity of reactants and the precise orientation, together with the effect of microheterogenization discussed above, lead to the outstanding performance of an enzyme as catalyst (examples are given in Table 1-2). Often, transient covalent bonds are formed between substrate and enzyme or coenzyme (see below) during a catalytic cycle. Serine, cysteine, histidine, lysine, aspartate or glutamate may donate en electron pair to a substrate, forming a covalent linkage as shown in Fig. 1-7 for the well-known charge-relay system in serine proteases. The highly reactive intermediates formed may be attacked by water or a second substrate to yield the characteristic products of the reaction.

1.3 Cofactors and Coenzymes

The chemical potential of side chains found in amino acids is limited; for example, there are no efficient electron acceptors. Therefore, enzyme catalysis incorporates if necessary additional chemical potential by specific metal ions, for example, Zn^{2+} (see Fig. 1-6), Fe^{2+} Co^{2+}, Cu^{2+} and others Examples are shown in Fig. 1-8 for the coordination of the transition metal ions in protein structures. Besides metal ions, cofactors or coenzymes serve to activate groups and participate in the catalytic process. A summary of cofactors and coenzymes is given in Table 1-4; the relation to vitamins is quite apparent. Chemical structures are presented in Table 1-5. Coenzymes and cofactors may act by nucleophilic or electrophilic attack on the sub-

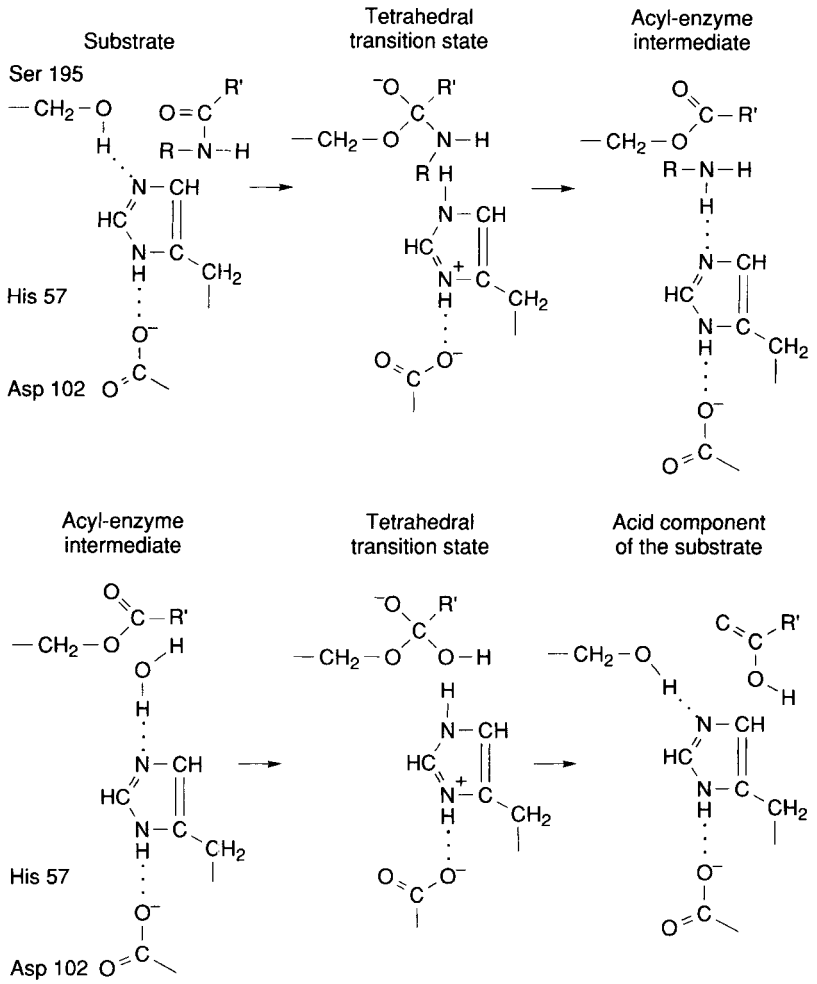


Figure 1-7. The catalytic triad in serine proteases. The reactive serine forms an acyl enzyme as a covalent intermediate during the proteolytic cleavage of a peptide bond. During substrate binding a proton is transferred from serine 195 to histidine 57, and the positive charge of the imidazole ring is stabilized by interaction with the carboxylate side chain of aspartic acid 102. The numbering corresponds to the structure of chymotrypsin.

strate(s) to initiate a reaction. Cofactors are tightly (covalently) bound to the protein and may undergo cyclic reactions during the catalytic process but will return to the ground state at the end. If oxygen is the terminal electron acceptor in FAD, FMN or $NAD(P)^+$ linked reactions, these cofactors require a second reaction with the co-substrate oxygen to regenerate the active form. In the older literature cofactors sometimes are called "prostetic groups". Coenzymes are bound in an association/dissociation equilibrium to enzymes and have to be present in sufficient concentration to obtain maximal enzymatic activity. Some are regenerated in the catalytic cycle

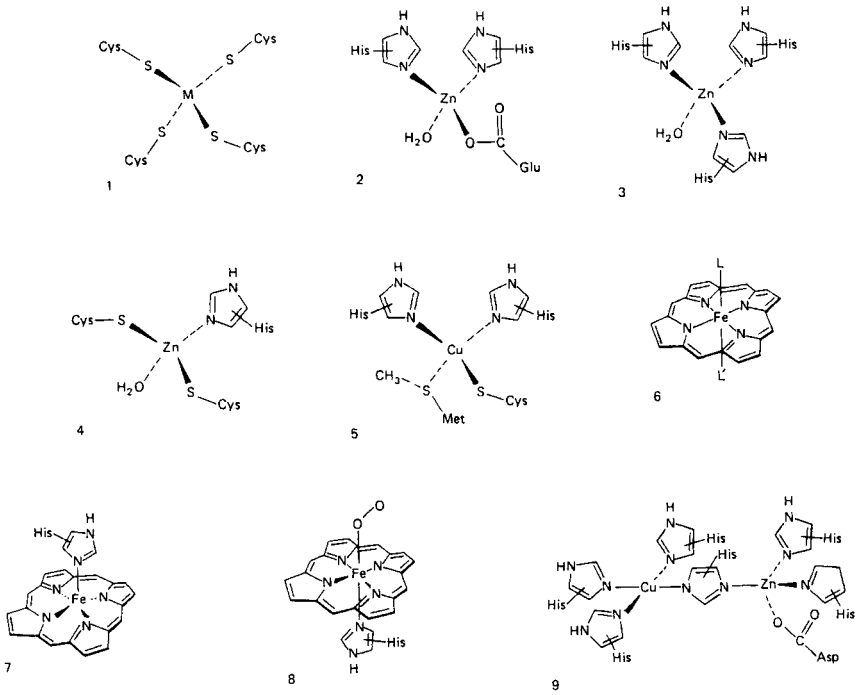


Figure 1-8. Typical co-ordination complexes of transition metal ions in proteins. 1: M may be Fe^{2+}, as in rubredoxin, or Zn^{2+} as in aspartate transcarbamylase and alcohol dehydrogenase, 2: carboxypeptidase A, 3: carbonic anhydrase, 4: liver alcohol dehydrogenase, 5: azurin, 6: heme group, L is His and L'either His or Met in cytochromes, 7: deoxy-heme group in hemoglobin and myoglobin, 8: oxyform of 7, 9: superoxide dismutase.

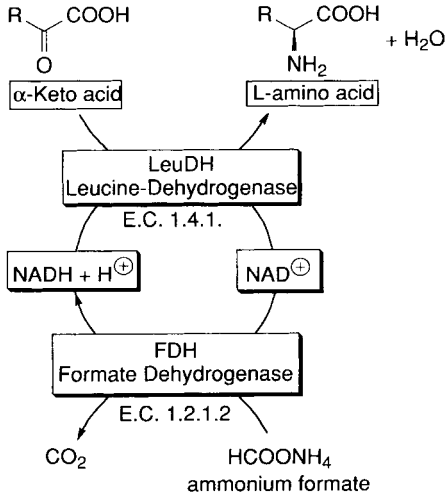


Figure 1-9. NADH regeneration using formate dehydrogenase (FDH) in a coupled reaction with leucine dehydrogenase (LeuDH).

Table 1-4. Cofactors and coenzymes.

Compound[a]	Function	Relation to vitamins
$NAD^+/NADH + H^+$	redox reactions and hydrogen transfer	vitamin PP, (niacin)
$NADP^+/NADPH + H^+$	redox reactions and hydrogen transfer	vitamin PP, (niacin)
FAD	redox reactions and hydrogen transfer	vitamin B_2, (riboflavin)
FMN	redox reactions and hydrogen transfer	vitamin B_2, (riboflavin)
Haem	transfer of electrons	–
Coenzyme A	transfer of acyl groups	pantothenic acid
ATP	metabolic energy, phosphate-, pyrophosphate transfer, adenylation	
Pyridoxal phosphate (PLP)	transamination, amino acid decarboxylation	vitamin B_6, (pyridoxine)
Thiamine pyrophosphate (TPP)	decarboxylation, transfer of C_2 units	vitamin B_1, (thiamine)
Biotin	transfer of CO_2	biotin
Tetrahydrofolic acid	transfer of C_1 groups	folic acid
S-Adenosyl methionine (SAM)	methylation	–
Adenosyl-cobalamine	isomerisation (hydrogen-shift)	vitamin B_{12}
Methyl-cobalamine	methylation	cyano-cobalamine

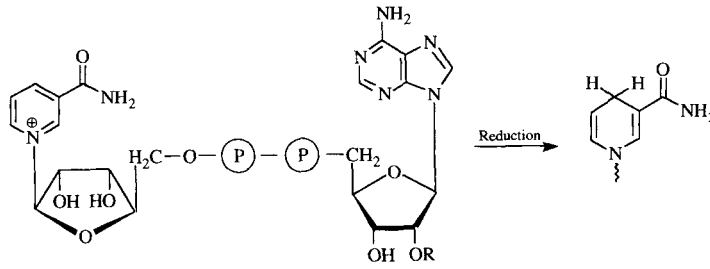
a The structure of the various compounds is summarized in Table 5.

while bound to the enzyme, for example, pyridoxal phosphate or thiamine pyrophosphate, so that catalytic amounts are sufficient to sustain the reaction. Others require one or more separate reactions with cosubstrates other than oxygen to regenerate the starting material. This holds true for example for $NAD(P)^+$, NAD(P)H, SAM, coenzyme A, ATP and other nucleotide triphosphates. In such instances, the coenzyme is consumed in stoichiometric relation to product formation. This relation may render enzymatic synthesis quite expensive unless efficient coenzyme regeneration cycles can be devised. *In situ* regeneration processes have been successfully developed in recent years, especially for the nicotinamide nucleotides. The stoichiometric relation with product formation is shifted from the expensive coenzyme to the conversion of a cheap cosubstrate such as formate, as shown schematically in Fig. 9. A detailed discussion of coenzyme regeneration is found in another chapter.

Table 1-5. Chemical structures of cofactors and coenzymes.

Nicotinamide nucleotides

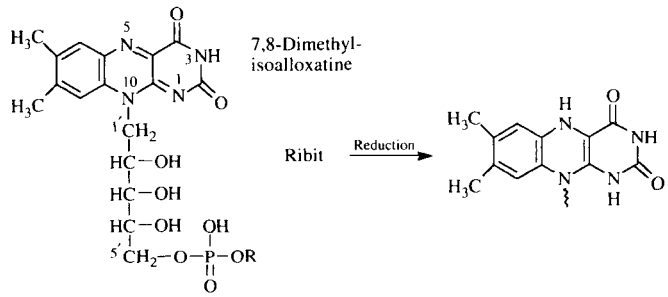
NAD^+ and $NADP^+$ and their reduced forms are involved in many dehydrogenase reactions within the cell. They are water-soluble, and are usually free to diffuse away from the enzyme, after conversion to the oxidized or reduced form to take part in another dehydrogenase reaction catalyzed by another enzyme.



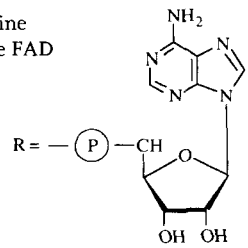
R = H: $NAD^{\oplus}$
R = PO_3H: $NADP^{\oplus}$
Ⓟ = Phosphate (PO_3H_2-group)

Flavin nucleotides

FAD is the coenzyme of a class dehydrogenases called *flavoproteins*. The flavin moiety of the molecule is derived from riboflavin (vitamin B_2). Reduction of FAD involves the two unsubstituted N atoms of the isoalloxazine structure.



Flavin adenine dinucleotide FAD



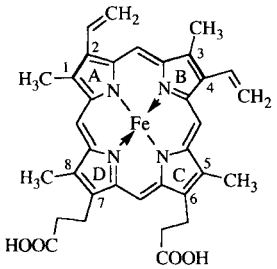
Flavin mononucleotide
FMN R = H

Table 1-5. (cont.).

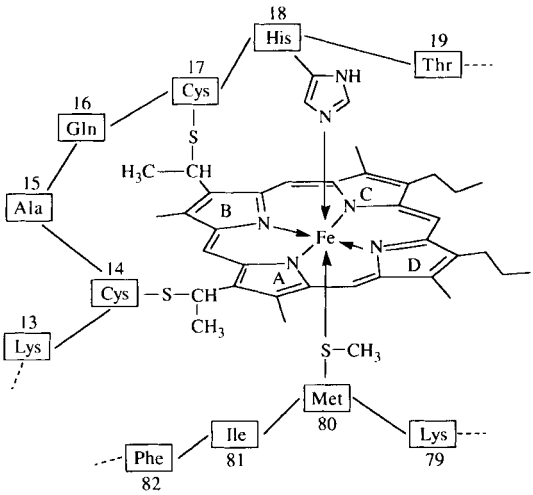
The electron transport chain

Enzymes in the electron transport chain split hydrogens into H^+ and e^-. The electrons are then carried by enzymes called cytochromes a, b, c, d.

These enzymes are able to accept an alectron and then pass it on to another cytochrome. The iron atom is bound, within the haem a, b, c, d group, to a porphyrin coenzyme identical with that found in haemoglobin, with the difference that in the cytochromes the iron undergoes oxidation and reduction.



Haem b



Cytochrome c with haem c

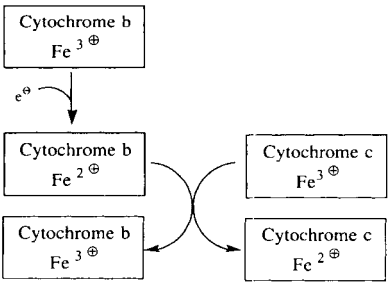
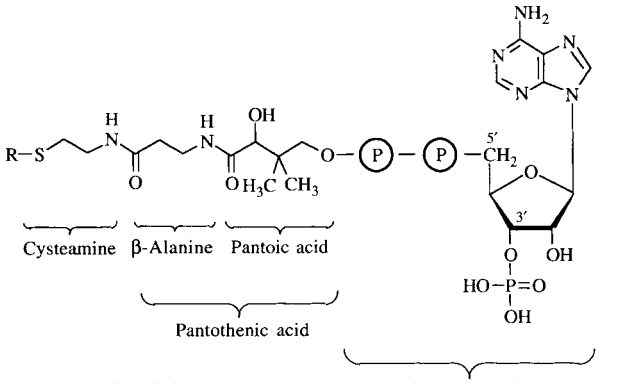


Table 1-5. (cont.).

Coenzyme A (CoA-SH)

Coenzyme A is a complex molecule which contains a free sulfydryl (–SH) group. This group can react with a carboxyl group to form a thioester. In acetyl CoA, the thioester linkage can activate the methyl carbon as well as the acetyl group.



R = H: CoA-SH
R = Acetyl: Acetyl CoA

Adenine nucleotides

ATP, ADP and AMP are coenzymes influencing the direction of flow in metabolic pathways. In addition ATP often functions as a donor of a phosphate to other molecules in reactions catalysed by kinases.

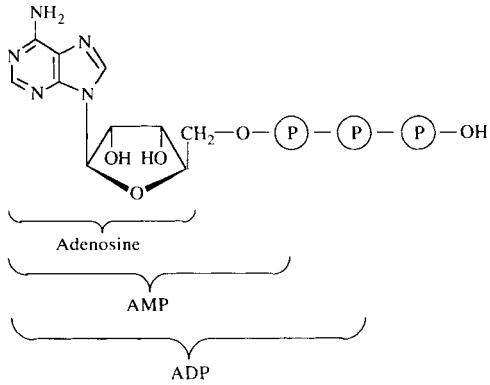
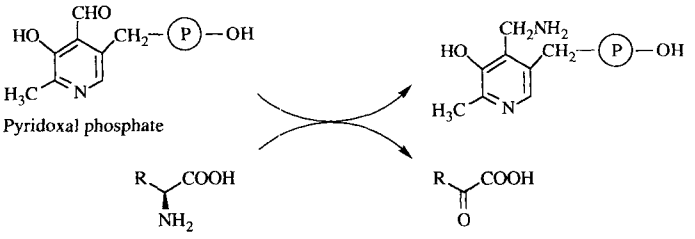


Table 1-5. (cont.).

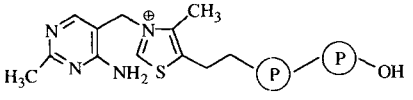
Pyridoxal phosphate

Pyridoxal phosphate, a derivative of vitamin B_6, acts as coenzyme in transamination and decarboxylation reactions. In a transamination reaction the aldehyde group of pyridoxal phosphate first forms a Schiff base with the amino group of the amino acid, which is then converted to keto acid. Pyridoxal phosphate is thereby converted to pyridoxamine phosphate which transfers the amino group to another keto acid to form the corresponding amino acid.



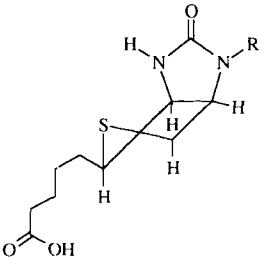
Thiamine pyrophosphate

All biochemical reactions with participation of thiamine start with C–C-bond cleavage of 2-oxo carbonyl-compound and proceed with formation of an "activated aldehyde", TPP catalyzes decarboxylation of α-keto acids, oxidative decarboxylations together with lipoic acid, and transketolase reactions.



Biotin

Biotin containing enzymes catalyze CO_2-transfer reactions: these are carboxylation, transcarboxylation and decarboxylations. The carboxy group of biotin is bound to an ε-NH_2 of lysine in an enzyme protein.



R = H: *D* (+)-Biotin
R = COOH: *N*-1′-Carboxybiotin

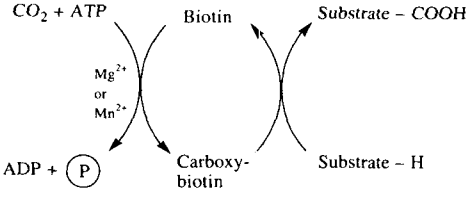
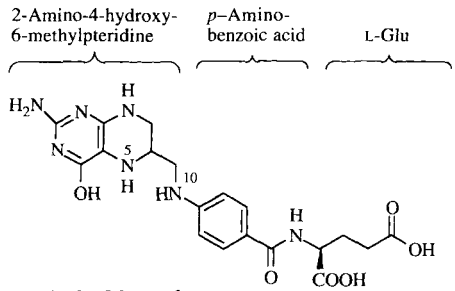


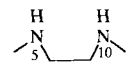
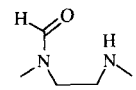
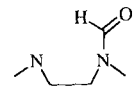
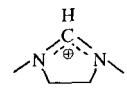
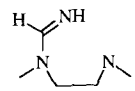
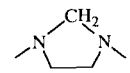
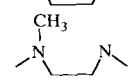
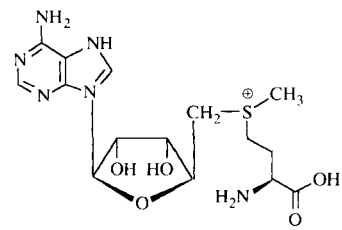
Table 1-5. (cont.).

Folate coenzymes

The transfer of a C_1-group like methyl, methylene, formyl or formimino often involves folic acid in one of its substituted forms



Tetrahydrofolic acid

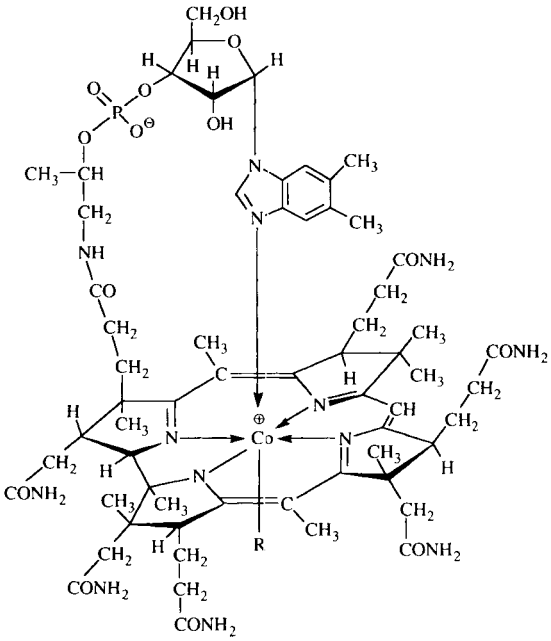
Compound	Structure	C_1-fragment
Tetrahydrofolic acid		–
N^5-formyl-		formic acid
N^{10}-methenyl-		formic acid
N^5, N^{10}-methenyl-		formic acid
N^5-formimino-		formic acid
N^5, N^{10}-methylene-		formaldehyde
N^5-methyl-		methanol
***S*-Adenosyl-l-methionine**		

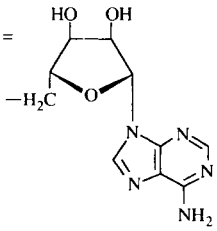
S-Adenosyl-L-methionine as sulfonium compound could transfer its methyl group as $CH_3^{\oplus}$ to nucleophile centers of substrates in biochemical reactions.

Table 1-5. (cont.).

Cobalamine

Adenosyl-cobalamine catalyzes hydrogen shifts as a special isomerisation reaction. With exception of reduction of ribonucleotides the H-shift occurs intramolecularly. Methyl-cobalamine and tetrahydrofolic acid are the coenzymes in methylating homocysteine to methionine.



R =  : Adenosyl-cobalamine

R = CH_3 : Methyl-cobalamine

R = CN : Cyano-cobalamine (Vitamine B_{12})

1.4 Enzyme Nomenclature

The IUB has classified enzymes into 6 main classes according to the type of reaction catalyzed:

1. **Oxidoreductases**
   These catalyze oxidation/reduction reactions, transferring hydrogen, oxygen, and/or electrons, between molecules. In this important class belong dehydrogenases (hydride transfer), oxidases (electron transfer to molecular oxygen), oxygenases (oxygen transfer from molecular oxygen), and peroxidases (electron transfer to peroxide)
2. **Transferases**
   These catalyze the transfer of groups of atoms, e.g. amino-, acetyl-, phosphoryl-, glycosyl- etc. from a donor to a suitable acceptor. Reactions covered in class 1, 3, or 4 are excluded.
3. **Hydrolases**
   These catalyze the hydrolytic cleavage of bonds. Many commercially important enzymes belong to this class, e.g. proteases, amylases, acylases, lipases, and esterases.
4. **Lyases**
   These catalyze the non-hydrolytic cleavage of, for example, C – C, C – O or C – N bonds by elimination reactions leaving double bonds or, in reverse, adding groups to a double bond. Examples are fumarase, aspartase, decarboxylases, dehydratases, and aldolases; many lyases are important catalysts for organic synthesis. In older literature class 4 enzymes are often called synthases, e.g. tryptophan synthase. These should not be confused with synthetases, as class 6 enzymes are sometimes called.
5. **Isomerases**
   These catalyze isomerization and transfer reaction within one molecule. The most prominent member of this group is D-xylose ketol-isomerase, commonly known as glucose isomerase.
6. **Ligases**
   These catalyze the covalent joining of two molecules coupled with the hydrolysis of an energy rich bond in ATP or similar triphosphates. An example is γ-L-glutamyl-L-cysteine: glycine ligase (ADP-forming), also found under the name glutathion synthetase. Ligases find limited applications only for synthetic purposes.

The main classes are further subdivided into subclasses and subgroups, as in part indicated above. A complete ordering system can be found in the publications from IUB. The systematic name of an enzyme is based on the equation of the chemical reaction taking place and the type of reaction, followed by the suffix-ase. By international agreement the catalytic reaction is expressed and identified by 4 groups of numbers according to the E.C. (enzyme classification) system introduced above. For example, an enzyme converting an alcohol to an aldehyde (or ketone) using NAD as coenzyme would be classified as

oxidoreductase	main class 1
acting on CH – OH groups	sub class 1.1
using NAD^+ as acceptor	sub group 1.1.1
alcohol: NAD^+-oxidoreductase	E.C. 1.1.1.1

The last number is the serial number of an enzyme identified by the first three entries. An alcohol could be converted to similar products also using oxygen as the electron acceptor by an oxidase.

oxidoreductase	main class 1
acting on CH – OH groups	sub class 1.1.
using oxygen as acceptor	sub group 1.1.3
alcohol: oxygen-oxidoreductase	E.C. 1.1.3.13

For newly isolated enzymes the nomenclature committee of IUB assigns the correct E.C. number to avoid confusion. The last edition (1992) contains 3196 entries. This code system is used in the scientific literature, textbooks and catalogues to identify an enzyme on the basis of the chemical reaction it catalyzes. For a proper description the source has to be included. Besides the systematic name IUB also lists trivial names or recommended names, the two enzymes described above being better known as alcohol dehydrogenase or alcohol oxidase, respectively. The recommended name is shorter and preferred in discussion after the catalyst has been duly identified. It should be noted that the classification is not based on the enzyme source and in general not on a single substrate. The physical properties of the individual enzyme protein may vary, for example, pH optimum, K_m values, stability, substrate range etc., but the systematic name and the number code are identical as long as the same type of reaction is catalyzed. Often it is worthwhile to test enzymes from different sources for the reaction of interest to find the optimal catalyst. Numerous successful applications of enzymes are described in the following chapters. Many more opportunities exist for innovative approaches in synthetic chemistry.

1.5 Enzyme Kinetics

1.5.1 Reaction Rate and Substrate Concentration

An enzymatic reaction may be described by the following steps: first, binding of enzyme E and substrate S occurs; second, while bound to the enzyme the substrate will be converted to the product P; finally, the product is released from the enzyme and free enzyme becomes available for the next cycle. In the simple case of a one substrate reaction this can be described by the following equations

$$[S] + [E] \underset{K_1^{-1}}{\overset{K_1}{\rightleftharpoons}} [ES] \rightleftharpoons [EP] \overset{K_2}{\rightleftharpoons} [E] + [P]$$

Michaelis and Menten derived a mathematical description for the reaction rate of an enzymatic process from this scheme

$$V = V_{max}\frac{[S]}{K_m + [S]} \quad (1)$$

$$V_{max} = K_2[E] \quad (2)$$

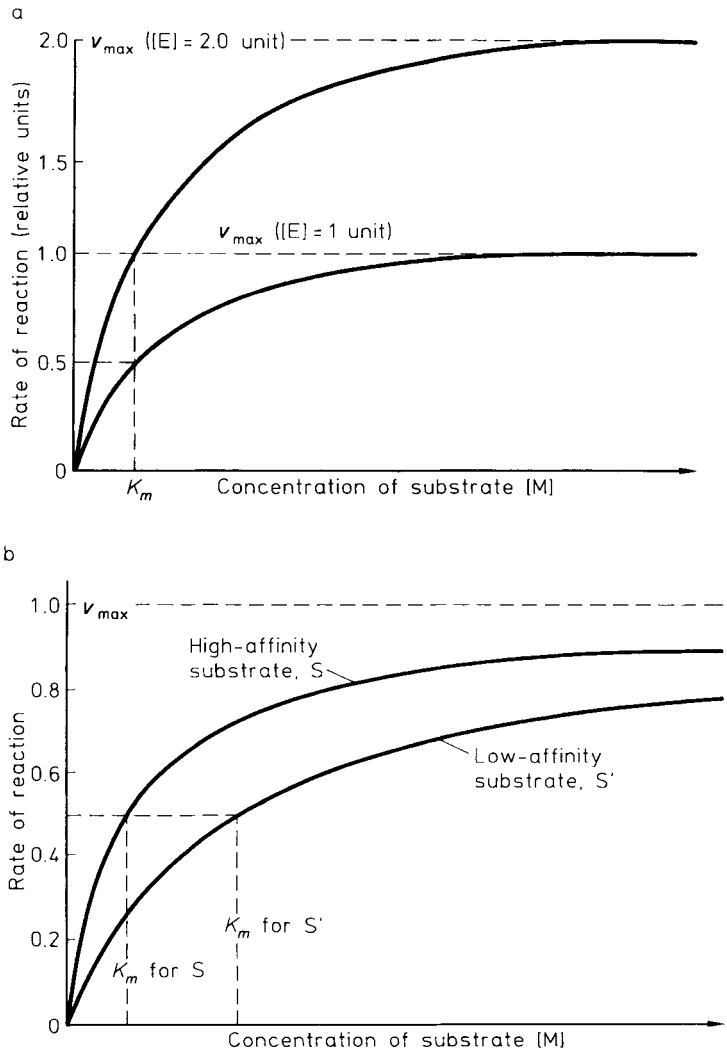


Figure 1-10. Reaction rate as a function of substrate concentration: a) using two different enzyme concentrations in the assay, b) comparing low and high affinity substrates of the same enzyme.

with the assumption that the binding of substrate and enzyme is reversible and fast compared to product release. Equation (1) represents a hyperbolic curve, relating reaction rate with substrate concentration as shown in Fig. 1-10. The hyperbola is described by two parameters: V_{max} and K_m. K_m the so-called Michaelis constant, is defined as the substrate concentration for which the observed reaction rate is half of V_{max}. The K_m value characterizes the affinity between substrate and enzyme and in a first approximation can be viewed as the dissociation constant of the enzyme-

substrate complex ES. K_m is independent of enzyme concentration and usually has values between 10^{-6} and 10^{-2} M. V_{max} is the maximal reaction rate possible if every enzyme molecule present is saturated with substrate and is a property of the particular enzyme. It may be related to the molecular mass of the enzyme and then is called turnover number, representing the number of substrate molecules converted per active site of an enzyme molecule per unit of time. The turnover number may have values between 10^{-3} and 10^{6} s^{-1}; 10^{3}–^{4} s^{-1} is commonly found.

Another quantity used frequently for the characterization of an enzyme is the catalytic activity. The unit for the catalytic activity is the Katal (kat), as defined by the International Union of Biochemistry (IUB), 1 kat corresponds to the amount of enzyme catalyzing the conversion of one mole of substrate per second at 30 °C under specified conditions. In the biochemical literature, another quantity is often used, the international unit (IU); 1 IU catalyzes the conversion of 1 μmole of substrate per minute under specified conditions. From the catalytic activity other values such as volumetric activity [kat l^{-1}; IU ml^{-1}] or specific activity [kat kg^{-1}; IU mg^{-1}] are derived. Catalytic activity can be determined unequivocally even in crude mixtures and if the molecular properties of the enzyme are unknown. Therefore, enzymes are quantified measuring their catalytic activity and sold on the basis of activity. To ensure reproducible and meaningful results when measuring enzyme activity, several points have to be taken into consideration. As shown in Fig. 1-10 [Eq. (1)], the reaction velocity depends on substrate concentration; for [S] ≥ 100 K_m the reaction rate becomes zero order and so no longer depends on substrate concentration. In special cases, for example, lipases reacting at an interface, the reaction rate depends on the available interface rather than the concentration. Lipases are therefore preferentially analyzed in stable emulsions. The catalytic activity has to be determined at sufficiently high substrate concentration (≥ 10 K_m) to ensure pseudo-zero order rates. This may be difficult to achieve with substrates of low solubility. Furthermore, it is desirable to measure initial rates, when only a small amount of total substrate is converted; [S] remains essentially constant during the reaction time and [P] is small. In reactions involving more than one substrate all concentrations have to be considered. If an unknown substrate or reaction is investigated two or more substrate levels should be employed. At low substrate concentration and high K_m values the observed reaction rate may be small and not easily differentiated from background noise, while, at high substrate concentration, inhibition by surplus substrate (see below) may cause a substantial drop in the rate.

The reaction rate is best determined by analyzing product formation as a function of time by physical methods such as UV/VIS spectroscopy, optical rotation, potentiometry, etc. Alternatively, formation of a coproduct produced in stoichiometric relations may be followed, such as formation of NAD(P)H in dehydrogenase reactions, which is followed conveniently at 340 nm in a spectrophotometer. Product formation may be coupled to a second reaction using a surplus of an auxiliary enzyme producing an easily quantified signal, for example $(NAD(P)^{+}$ or $NAD(P)H+H^{+}$ with a dehydrogenase.

$$\text{L-glutamine} + H_2O \xrightleftharpoons{\text{glutaminase}} \text{L-glutamate} + NH_3$$

$$\text{L-glutamate} + NAD^+ + H_2O \xrightleftharpoons{\text{glutamate-dehydrogenase}} \alpha\text{-ketoglutarate} + NADH + NH_4^+$$

A similar approach determines a quinone-imine dye formed by the reaction of H_2O_2catalyzed by peroxidase.

$$2\ \text{ethanol} + 2O_2 + \xrightarrow{\text{alcohol oxidase}} 2\ \text{acetaldehyde} + 2\ H_2O_2$$

$$2\ H_2O_2 + \text{4-amino antipyrine} + \text{phenol} \xrightarrow{\text{peroxidase}} \text{quinone-imine dye} + 2\ H_2O$$

In such coupled systems, care must be taken in choosing reaction conditions, such that the enzyme of interest is catalyzing the rate-determining step. Special synthetic colorless substrates converted to colored products have been developed for hydrolases (esterases, phosphatases, glycosidases and proteases); 4-nitrophenol or 4-nitroanilide are used as the alcohol or amide component, which can be measured readily around 400–420 nm.

$$\text{4-nitrophenyl phosphate} + H_2O \xrightarrow{\text{phosphatase}} \text{4-nitrophenol} + \text{phosphate}$$

$$N\text{-}\alpha\text{-benzoyl arginine-4'-nitroanilide'}) \xrightarrow{\text{protease}} N\text{-}\alpha\text{-benzoyl arginine} + \text{4 nitroaniline}$$

If direct physical measurements are not available or feasible, the enzymatic reaction can be stopped at predetermined times by rapid heating, acid treatment, or similar measures and the amount of product present at time t measured by available analytical techniques such as HPLC, GC, TLC (with or without prior derivatization). Controls are required to ensure that the conditions employed to stop the enzymatic reaction do not destroy the product and that the derivatization is complete. It may be more convenient to follow the decrease in substrate concentration over time as a measure of enzyme activity. This has the disadvantage that the difference of two large values is prone to error. If such an approach is adopted it has to be proven by independent experiments that the anticipated product is actually formed.

1.5.2
Inhibitors and Effectors

Chemical compounds negatively influencing the reaction rate of an enzyme-catalyzed process are called inhibitors. Irreversible inhibitors might be reactive substrate analogs forming a covalent linkage to the enzyme after binding and in this way blocking the reactive site. Usually, such reactions are designed intentionally. Heavy metal ions present in trace amounts as contaminants in crude substrates may react with essential sulfhydryl groups and inactivate the enzyme. the situation is similar to the well known poisoning of a metal catalyst by sulfur compounds. Far more important for enzymatic processes are reversible inhibitors, forming specific enzyme inhibitor complexes and thereby influencing the reaction rate. It is important to note that substrates and especially products might inhibit an enzymatic

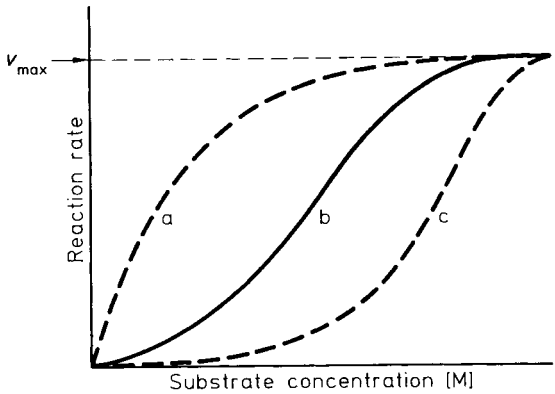


Figure 1-11. Reaction rate as a function of substrate concentration illustrating allosteric regulation of enzyme activity: a) rate in the presence of an allosteric activator, b) rate in the absence of effectors, c) rate in the presence of an inhibitory effector.

reaction as might substrate analogs. Inhibition by substrate and/or product(s) is important when considering how much of the activity added actually can be utilized at a given set of reaction conditions. Such reaction engineering aspects are treated in more detail in Chap. 4 of this book. Many enzymes may also be activated by inorganic ions such as Ca^{2+}, K^+, or Cl^- possibly raising V_{max} by stabilizing certain protein conformations. If such an effect is noted, the activator should be added in saturating amounts. Special effects are observed in the kinetics of allosteric enzymes. A typical sigmoidal curve describing reaction rate as a function of substrate concentration is presented in Fig. 1-11. Binding of an effector to the regulatory center alters the reaction rate very efficiently and subtly and is often used in nature to divert the metabolic flow into different directions at branching points. Such a response is important for living systems, but rarely will be seen with enzymes employed in organic synthesis. The complex kinetics may be described by appropriate mathematical models, found in the specialized literature.

1.5.3
Influence of pH and Buffers

Enzymes contain many polar amino acids at the surface which may be protonated or unprotonated depending on the pH of the surrounding medium. Typikal pK_s values are included in Table 1-3. Consequently, charges on the protein surface are altered and K_m and V_{max} will depend on pH. Fig. 1-12 illustrates that an optimum of the reaction rate is observed as a function of pH. The optimal pH may vary slightly for different substrates, reflecting differential binding energies. The pH optima for the forward and reverse reaction of the same enzyme are not identical and may differ by 2–3 pH units. In the laboratory, the pH is usually set and maintained using buffers. Selection of buffer ions may influence the observed reaction rate as shown in Fig. 1-3. The reasons are not well understood and are thought to be related to the polarity of buffer molecules interacting with the protein, influencing simultaneously hydratization and solubility of substrates. On the preparative scale, pH is maintained better by a pH-stat arrangement, saving chemicals and separation cost. Also, on the

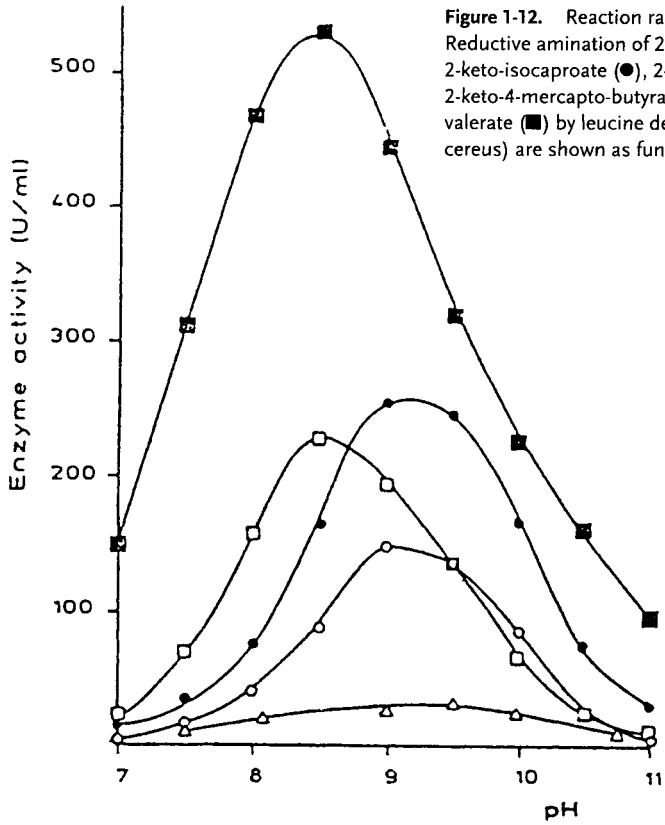


Figure 1-12. Reaction rate as a function of pH. Reductive amination of 2-keto caproate (○), 2-keto-isocaproate (●), 2-keto-valerate (□), 2-keto-4-mercapto-butyrate (△), and 2-keto iso-valerate (■) by leucine dehydrogenase (Bacillus cereus) are shown as function of pH.

preparative scale, high substrate levels are desired, impossible to buffer sufficiently in reactions involving release or consumption of protons.

In switching from buffered to pH-stat operation one should be aware of changes in kinetics as discussed above. The pH of the solution is important not only for enzyme activity but also for enzyme stability. Unfortunately, the optimal pH values for enzyme activity and stability are not necessarily identical, as is well documented in the literature for the hydrolysis of penicillin G by penicillin acylase. In such cases, the method for controlling pH and mixing behavior of the reactor may become crucial.

1.5.4
Temperature

Another important factor for enzyme activity is temperature. In general, the reaction rate will increase with temperature (Fig. 1-14). From an Arrhenius type plot, the activation energy of the process may be calculated. With increasing temperature, however, the mobility of protein segments increases while the strength of hydro-

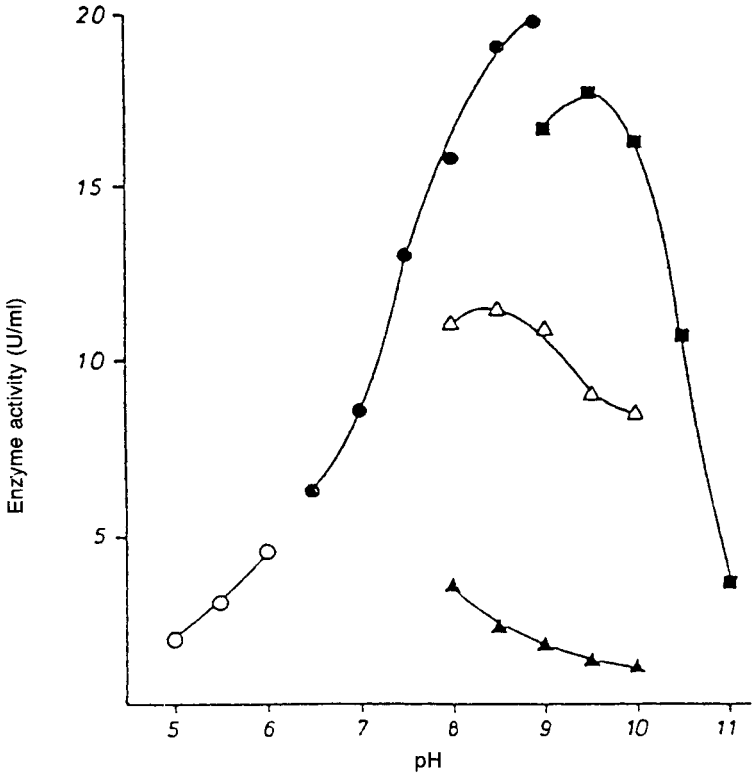
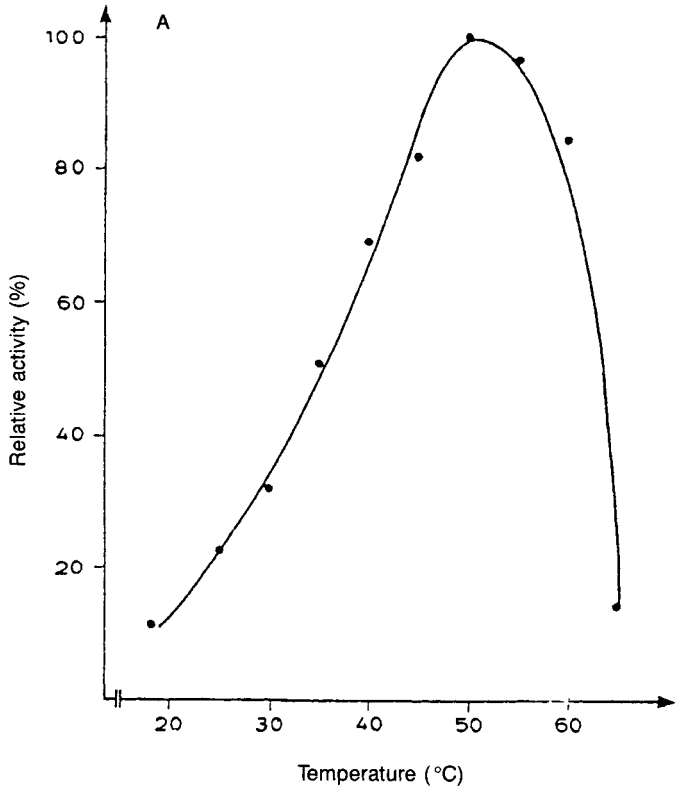


Figure 1-13. Effect of pH on the activity of *sec*-alcohol dehydrogenase (Candida boidinii) during the oxidation of isopropanol in various buffers in 50 mM concentration: ○ sodium citrate, ● potassium phosphate, △ triethanolamine/HCl, ▲ Tris/HCl, ■ glycine.

phobic interaction decreases. At first, this results in a decrease in catalytic activity, but, with further rise in temperature, in complete deactivation. Thermally induced denaturation of proteins often leads to aggregation which is not readily reversible. Denaturation may be expected in the temperature interval between 30 and 80 °C. The optimal temperature of operation has to be lowered if long reaction times or long service life of an enzyme are required. For enzyme assays, a defined (for example 30 °C) and constant temperature has to be maintained. Enzymes from extremely thermophilic microorganisms may be almost inactive at ambient temperatures and operate in the temperature interval between 80 and 120 °C.



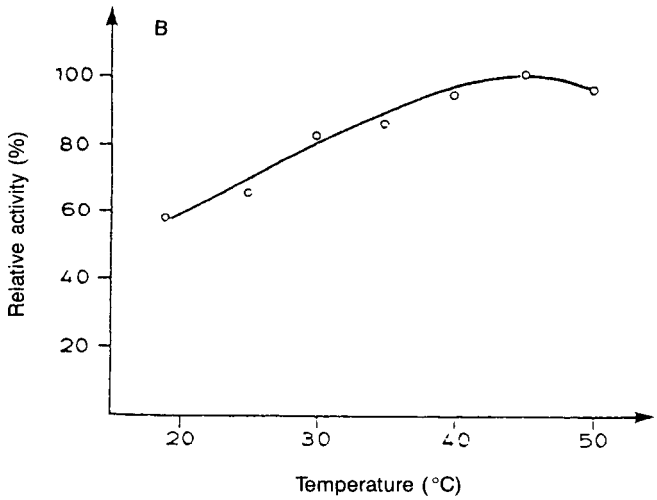


Figure 1-14. Temperature dependence of the reaction rate A: L-2-hydroxysisocaproate dehydrogenase *(L. confusus)* B: D-lactate dehydrogenase *(L. confusus)*.

1.6
Organic Solvents as Reaction Media

Enzymes as biocatalysts have been developed for aqueous reaction systems. Application of enzymes in the presence of organic solvents is of interest to organic chemists because substrates may not be sufficiently soluble in water, or the equilibrium of the desired reaction may be unfavorable in aqueous solution. The following general approaches are used:

- to add increasing concentrations of water miscible solvents to the reaction system,
- to work in two-phase systems composed of water and an immiscible solvent,
- to work in nearly anhydrous organic solvents with minimal necessary amounts of water.

In the first two cases, the enzyme may be employed either in the soluble state or immobilized. In nearly anhydrous organic solvents the enzyme is present in the solid state only. The presence of organic solvents will influence activity as well as stability of enzymes. In recent years, work of various groups has shown that the majority of bulk water in a reaction system may be replaced by organic solvents. A certain low amount of residual water is needed for activity; 0.02% may be sufficient. Organic solvents influence the dielectric properties of the reaction medium and to varying degree disrupt ordered water structures. This, in turn, will influence the non-covalent, weak forces responsible for the ordered structures of an enzyme. Protein structures may be stabilized by adsorption, crosslinking, or covalent binding to a hydrophilic surface. Immobilization may also help to avoid denaturation at the interface in two-phase systems. If an immobilized or solid enzyme preparation is used, it is important to provide sufficient surface area to catalyze the reaction. In nearly anhydrous systems, maintaining the pH in the optimal range is a problem. In such cases the enzyme has to be prepared (dried) under pH conditions providing the optimal activity. This way the dissociation of charged groups on the enzyme surface is fixed; there obviously exists a memory effect. The selection of a suitable solvent with regard to activity and stability may be guided by the log P concept, where P is the partition coefficient of the solvent in an octanol/water biphasic system. Hydrophilic solvents with log P values < 2 often lead to enzyme deactivation if present in high concentrations; in contrast, apolar solvents with log $P \geq 4$ are compatible with enzymes, leaving the essential layer of water molecules on the polar surface regions unperturbed. The results using solvents with intermediate values of log P (2–4) are unpredictable and depend very much on the individual case. Solvent selection and reaction conditions today are optimized empirically. Nevertheless, there are many examples which clearly demonstrate that enzymes can be employed successfully also in organic solvents.

1.7
Enzyme Handling: Quality Requirements

An increasing number of enzymes are offered on the market by manufacturers and vendors. Enzymes are produced for different purposes and may differ widely in purity and price. In general, enzymes are sold on the basis of unit of activity. The catalogue or data sheet of the supplier should contain information or a reference on assay conditions and definition of the unit. Special requirements for storage (4 °C or −20 °C) are recorded on the label. Enzymes are shipped as dry powders, suspensions or (frozen) concentrated aqueous solutions. The sample may contain undeclared additives such as inert materials (for example, fillers or filter aids), salts or saccharides for stabilization, or precipitating agents such as ammonium sulfate or polyethyleneglycol. Crude preparations may also contain several enzymatic activities, for instance crude pig liver esterase is a mixture of esterases, lipases and other enzymes. The presence of other proteins may be inferred from the specific activity (U/mg protein) of the enzyme preparation, provided the catalytic activity of the pure protein of interest is known. All common protein assays are relative measurements only and therefore depend to some extent on the method and the protein used for calibration. If desired, compounds of low molecular weight can be removed from the enzyme by dialysis, ultrafiltration or gel filtration. For assay purposes, the necessary dilution of the catalyst may often be sufficient to avoid interferences. Wetting of dry proteins is not without problems, resolubilization of (freeze) dried material may take many hours and appropriate controls should be applied if the expected conversion or activity is found to be low.

The requirements for purity of an enzyme are not very high from the point of view of application in the synthesis of fine chemicals. More importantly, enzyme quality can be accessed fairly accurately by establishing a mass balance. Only such activities responsible for undesired side reactions have to be removed. As an example, fumarase (adding water to fumaric acid) should be absent from an aspartase preparation (adding ammonia to fumaric acid), because the enzymatic reaction is performed in water. Aspartase, however, is "silent" in a fumarase preparation as long as ammonium ions are absent. Therefore, aspartase does not necessarily need to be removed from a catalyst employed for production L-malic acid. Very often one can exploit the fact that the reaction in synthetic applications is restricted to the available reactants. Such a situation is entirely different from analytical applications, where complex mixtures are introduced and the selectivity of the reaction depends solely on the catalyst. An enzyme should be purified only to the degree necessary for the application. Absence or low levels of proteases are also desired to protect the enzyme catalyst from degradation. Stability under operation conditions is usually more important for production than the initial cost of the enzyme. Stability will allow long reaction times at low enzyme levels or prolonged service life in continuous processes.

Genetic engineering techniques can be employed for the production of large amounts if a certain enzyme is needed for application as a catalyst. In addition, specialized knowledge is available to bring the desired enzyme to an appropriate

level of purity. Such developments will lower the cost for the catalyst considerably, which should be kept in mind when analyzing and evaluating process costs.

1.8
Biotransformation Using Whole Cells

1.8.1
General Aspects

Microbial cells can be employed also as biocatalysts to achieve a desired conversion instead of isolated, cell-free enzymes. In contrast to microbial cultivation exploiting the complex metabolism of cells to produce, for example, organic acids or antibiotica from sheap nutrients, biotransformation utilizes only one or a few enzymes to convert added educts to a desired product. Since many microbial enzymes will accept non-natural compounds biotransformation also gives access to many products not found in nature, while microbial cultivation yields only natural primary or secondary metabolites. The acetic acid generator developed in 1824 by C. Ham represents a suprisingly modern industrial biotransformation process oxidizing ethanol to acetic acid using strains of *Acetobacter* immobilized on beech shavings in a current of air. About 100 years later the biotransformation process for the production of R-1-phenyl-1-hydroxypropane-2-one, the key chiral intermediate in the synthesis of (1R, 2S) ephedrine was commercialized using *Saccharomyces cerevisiae* to catalyze a stereoselective acyloin condensation. At the same time regiospecific oxidation of D-sorbitol to L-sorbose by *Acetobacter suboxydans* or related species was developed as a key step in vitamin C production. Biotransformations have since revolutionized the production and availability of steroid hormones and steroid related drugs including contraceptives, cortisone, prednisone etc. The modification of the steroid ring system as well as other cyclic structures by various microbial cultures is well documented in the literature. From this information, selection of potentially useful strains is possible for hydroxylation, oxidation, dehydration, reduction or dehydroxylation reactions. For a successful process development beyond a few grams and scale-up to an industrial process extensive screening and biochemical engineering will be necessary demanding a close collaboration between biologists, chemists and engineers.

There are far more microorganisms available from culture collections than enzymes on the market. A list of important culture collections can be found in Table 1-6. Further information can be found on the internet:
http://wdcm.nig.ac.jp/wfcc/wfcc.html

Fortunately, the majority of known microorganisms are classified as non-pathogenic and harmless to humans and the environment. These can be handled safely in the normal laboratory. Strains with desirable properties identified from the literature can often be ordered from catalogues of culture collections, just like chemicals from a supplier. There may be more than one strain of a given species listed in the catalogues as there are many "John Brown" found in a telephone directory. It is

Table 1-6. Important collections of microorganisms in the public domain.

American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland 20852, USA
Centraalbureau voor Schimmelcultures (CBS), P.O. Box 273, Oosterstraat 1, NL-3740 AG Baarn
Japan Collection of Microorganisms (JCM) RIKEN, Wako, Saitama 351-01, Japan
Institute of Applied Microbiology (LAM), University of Tokyo, Yavoi 1-1-1, Bunkyo-ku, Tokyo 113, Japan
Culture Collection of the Institute for Fermentation (IFO), 17-85 Juso-Houmachi 2-chome, Yodogawa-ku, Osaka, Japan
Agricultural Research Service Culture Collection (NRRL), Northern Regional
Research Center, Agricultural Research Service, US Department of Agriculture, 1815 North University Street, Peoria, Illinois 61604, USA
National Collection of Industrial and Marine Bacteria Ltd., 23 St Machar Drive, Aberdeen AB2 1RY, UK
Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSM-Z), Mascheroder Weg 1b, D-38124 Braunschweig

advisable to search for the strain identified by the unique catalogue number that was originally employed. If the particular strain is no longer available or cannot be identified from older literature on should screen for the desired activity in a number of strains from the same species to find a good substitute.

Usually strains are mailed as lyophilized culture or as a freshly inoculated agar slant. The investigator has to master some basic skills to maintain and grow cells under aseptic conditions if he wants to utilize these biological resources. Detailed information can be found in text books of microbiology and laboratory manuals. For aerobic organisms (needing oxygen for growth) or microaerophilic organisms (tolerant against trace amounts of oxygen) a working knowledge of media preparation is required (useful compositions are found in the literature or in catalogues of culture collections) as well as sterilization techniques for media and equipment to remove or destroy all living cells by heat, microfiltration or chemical treatment before inoculation with a pure strain. Aseptic conditions have to be maintained when inoculating sterile media with pure strains and in sampling the culture later. This means that airborne particles containing contaminating microorganisms have to be excluded from the growing culture, which can be accomplished working in a laminar flow hood and using exclusively sterilized equipment in handling. The laminar flow hood provides a positive pressure of filtered air in the working area and was especially designed to allow easy handling under aseptic conditions. Aerobic microorganisms are grown in a capped Erlenmeyer flask with baffles on a rotary shaker at constant temperatures in the range between 25 and 40 °C. Microaerophilic cultures are purged with nitrogen and agitated slowly if at all. Growth is observed as turbidity; the uniformity of the culture is checked using a light microscope. Pure cultures can be used to prepare seed stocks taking aliquots at the mid or late

logarithmic growth phase and freezing the suspension at −20 °C or −80 °C if available. Glycerol (5–10 %) or dimethylsulfoxide (2.5-5 %) may be added as a protecting agent. Working cultures can be maintained on nutrient agar in a Petri dish or vial at 4 °C for some weeks. There are numerous other methods to preserve and maintain pure cultures. The culture collection or a microbiologist should be consulted in case of doubt.

Anaerobic microorganisms cannot utilize oxygen as a terminal electron acceptor during growth and usually are very sensitive to oxygen in the environment. Instead they use a vast variety of organic and inorganic compounds as electron donors and acceptors in their energy metabolism. Strictly anaerobic strains are difficult to handle especially on a small scale without proper training and specialized equipment. Nevertheless anaerobic strains may contain interesting biocatalysts also for biotransformations, especially unusual redox enzymes.

Some microorganisms produced as starter cultures in the food industry can be easily obtained without necessitating the ability to grow microbial cultures under defined conditions in the laboratory. The most notable example is "baker's yeast", *Saccharomyces cerevisiae*, which can be bought cheaply in large amounts from the regional supplier of bakeries or in smaller amounts from any local food store. Baker's yeast is sold as pressed filter cake (usually containing a certain amount of starch granules), in the form of blocks or flakes or as a lyophilized preparation. The latter is rehydrated to a viable culture using water or buffer.

Because of the ready availability and cheap price, baker's yeast has been extensively used for the stereoselective reduction of carbonyl groups in numerous examples. Problems may arise from genetic and physiological differences in local strains of *S. cerevisae* beyond the control of the organic chemist. This leads to variation in the stereoselectivity and sometimes poor reproducibility of published results. The cells often contain more than one dehydrogenase/reductase accepting the substrate of interest and the result obtained is the sum of all parallel reactions. Besides, the biosynthesis of enzymes in general is strictly regulated in microorganisms. The activity of an enzyme in a given microbial cell may vary more than 1000-fold and depends on available nutrients and inducing agents, growth conditions, time of harvest, condition of storage etc. This biological variability (and the reasons behind them) should be kept in mind when planning and judging biotransformations. The relative merits of using whole cells versus cell-free enzymes as biocatalyst are summarized in Table 1-7.

Obviously whole cell biotransformations are the method of choice if the enzyme involved is not stable enough in isolated form or is an integral part of the cell membrane and utilizes the electron transport chain in complex coenzyme regeneration schemes. Whole cell biotransformations are particularly important for hydroxylation and oxidation reactions involving mono- and dioxygenases or epoxidases. Depending on the type of the reaction investigated, whole cells are employed as growing culture, as viable resting cells or in a nonviable form simply as a "bag of enzymes".

Table 1-7. Advantages and disadvantages using isolated enzymes or whole cell biocatalysts.

	Advantages	Disadvantages
Isolated Enzymes	Catalyst concentration as free process variable	Limited stability
	High catalyst concentration possible	Cofactor regeneration needed
	No side reaction	
	Simple product recovery	
	No transport limitation	
	Multienzyme reactions possible	
Whole cell biocatelysts	Unlimited availability exploiting growth	Side reactions
	Cofactor recycling by cellular machinery	Transport limitation
	Multistep conversions possible	Complex product recovery
		In general low space time yield

1.8.2
Biotransformation with Growing Cells

When growing cells are to be used for a biotransformation, e.g. of steroids, the cells are grown in a suitable growth medium, usually in batch culture. After an initial lag phase the cell will grow and multiply exponentially as observed by the turbidity of the culture. After one or more compounds become rate limiting for growth a period of linear growth may be observed before a stationary phase is reached, where viability of cells is maintained but growth stops. Eventually cells will die if energy sources are exhausted. Many fungi but also bacteria produce spores in the stationary phase, which are able to withstand adverse conditions and secure the survival of the species in nature.

The biosynthesis of a desired enzyme is often not parallel with growth: the specific activity will vary with the growth phase. Therefore timing of educt addition is crucial for product yield. The optimal conditions are usually determined experimentally. It may be advantageous to add small amounts of educt in the early logarithmic phase to help to induce the formation of the desired enzyme(s). Toxic substances are added best in the late logarithmic growth phase to minimize negative effects on growth and enzyme production. Repeated dosing helps to maintain low stationary levels of toxic or inhibitory educts but allow product accumulation to high levels provided the product is non-toxic. After batch or fed batch biotransformation processes, the biomass is separated and discarded after the conversion is completed or when side reactions become prominent. The product has to be isolated from whole broth or the spent medium depending on the yield. Fresh biomass has to be prepared from culture stocks for each successive experiment.

1.8.3
Biotransformation with Resting Cells

Resting cells are non-growing viable cells retaining many enzyme activities of growing cells. Baker's yeast, discussed above, essentially consists of resting cells of *S. cerevisiae*. In the laboratory, resting cells are obtained by growing the selected microbial culture under appropriate conditions until a high or maximal enzyme activity is reached in the cells. At this point in the growth cycle, cells are separated from the growth medium by centrifugation or filtration and washed with saline. Then cells are resuspended in the biotransformation buffer, and the conversion is followed by suitable analytical techniques.

Cell concentration can be varied and a higher catalyst concentration applied in comparison with experiments using growing cells. The addition of small amounts of glucose or other energy sources helps to maintain the electrochemical potential across the cell membrane and the viability of resting cells. If the biotransformation step requires coenzyme regeneration, co-metabolites such as glucose or glycerol have to be added in sufficient amounts. Resting cells are convenient to use as one large cultivation yields a uniform biocatalyst for many parallel biotransformations. It may also be possible to store the cell cake at 4 °C for some days or weeks without detrimental losses in activity. Otherwise resting cells may be conserved by lyophilization using sucrose or trehalose as a protectant. The biotransformation buffer is less complex than spent medium, and thus product isolation is usually easier than from growing cells. Once the initial growth is completed the danger of infection is also less using resting cells because biotransformation buffers often lack essential nutrients for growth. This makes handling in the laboratory more convenient.

1.8.4
Biotransformations with Permeabilized or Dried Cells

Microbial cells are separated from their surroundings by complex cell walls and one or more membranes. The cell wall provides the mechanical strength to withstand sudden changes is osmotic pressure while the membrane serves as an effective diffusion barrier. In addition, the membrane is important for the selective interaction of the cells with the environment and the maintenance of an electrochemical potential important for viability. Cell membranes may cause more or less severe mass transfer limitations, hindering educts from reaching biocatalysts inside the cells and the transport of products out of the cells. For lipophilic, uncharged and sufficiently soluble educts, passive diffusion across the membrane(s) may provide satisfying reaction rates. Detergents or solvents can be used to enhance permeability and mass transport. This approach should be used with care in case membrane-bound enzymes or coenzyme regeneration *via* the electron transport chain are necessary for the particular biotransformation. Otherwise, the integrity of the membrane may be of no concern for simple reactions involving only intracellular enzymes. For example L-malic acid is produced from fumarate using *Brevibacterium ammoniagenes* as a biocatalyst. The cells are permeabilized by treatment with bile to

improve transport of the charged reactants, and in addition a side reaction, the conversion of fumarate to succinate by membrane-bound enzymes of the citric acid cycle, is abolished. Resting cells may be dried by treatment with a large excess of cold (−20 °C) acetone; the resulting "acetone powder" can be stored at −20 °C for many months, yielding a convenient enzyme source. It should be noted that acetone treatment removes lipids from the cell membranes, and therefore membrane-bound activities are irreversibly damaged. Disturbance of membrane function is the major factor in the toxicity of solvents towards microbial cells and needs to be considered in a case-by-case evaluation.

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2
Production and Isolation of Enzymes

Yoshihiko Hirose

2.1
Introduction

This chapter gives a brief review of the isolation and production of enzymes. More detailed information can be obtained from various published textbooks and reviews[1–5]. Most of the industrial enzymes used for chemical synthesis are supplied in a crude form with an active enzyme content of only a few percent. The other constituents are inorganic salts, polysaccharides and diatomaceous earth used as stabilizers and excipients. Purified enzymes for biotransformation are supplied by some manufacturers in a crystal or immobilized form. These enzymes, though expensive, are easy to apply for biotransformation in organic media. The use of more purified enzymes is increasing.

Barriers to the production of industrial enzymes include economic factors, the availability of optimal enzymes and safety issues. Common fermentation and purification processes are described in Figs. 2-1 to 2-3. The process differs for extracellular and intracellular enzymes, liquid and solid culture, and enzyme application. The fermentation conditions are computer-controlled for optimization, e.g. of temperature, pH, agitation speed, aeration, demand oxygen etc.

There are no internationally standard assay methods for industrial enzymes and the definition of enzyme activity unit is also different for each enzyme. The activity of industrial enzymes is shown by various methods depending on manufacturers. For instance, commercial lipase activities are measured by the hydrolysis of olive oil under the various conditions and these figures are not comparable with each other. When customers apply these biocatalysts for chemical synthesis in organic solvents, these figure are sometimes reliable, and sometimes not. Users should not judge commercial enzymes based only on price and the activity shown in the table the manufacturer provides. Enzymes should be evaluated based on their practical performance under the conditions used. Most users of biotransformation are not experts in measuring enzyme activity, so the establishment of an assay method and practice are essential if one is to optimize the performance of enzymes.

Several commercial enzymes are powders including diatomaceous earth or

Preparation of fermentation medium ----- Starch, sugar, soybean powder, yeast extracts, minerals, inducer, etc.

Dissolution

Sterilization

Inoculation

Fermentation in flask (100–500 mL)

Partial inoculation

Fermentation in jar (3–30 L)

Partial inoculation

Fermentation in seed tank (1000 L–3000 L)

Partial inoculation

Fermentation in main tank (> 3000 L)

Broth-out

Filtration

Concentration by evaporation or ultrafiltration

Precise filtration

Solvent precipitation

Filtration or centrifugation

Drying

Product (crude product)

Dissolution

Ion-exchange chromatography

Salting-out

Desalting

Hydrophobic chromatography

Gel filtration

Desalting

Freeze-drying

Product

Figure 2-1. Common production process for industrial extracellular enzymes.

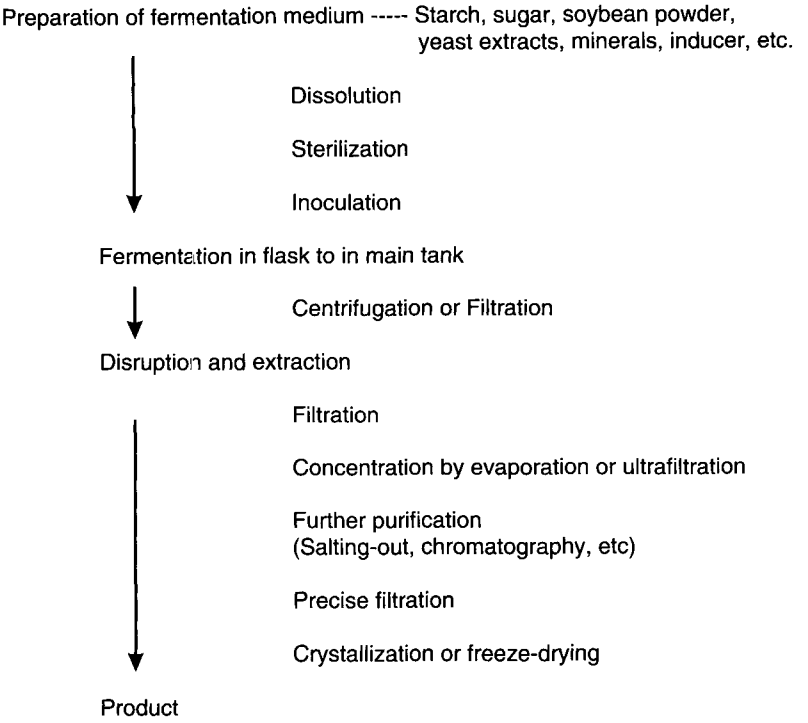


Figure 2-2. Common production process for special intracellular enzymes.

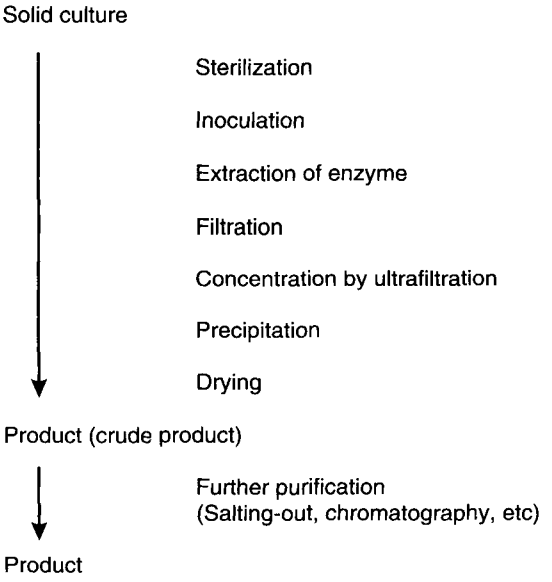


Figure 2-3. Common production process for special enzymes by solid fermentation.

dextrin. These enzymes should be used after immobilization on a suitable carrier. The activity of an immobilized enzyme usually is enhanced up to tenfold.

Regulatory assessments for enzymes used in biotransformation are not clearly stipulated. At present, food assessments of microbial enzymes are provided by AMFEP, which has suggested microbial enzyme purity and immobilization as given below.

Purity
A chemical and microbial specification must be given. Based on FCC recommendations, AMFEP recommended the following.

Arsenic	3 ppm
Lead	10 ppm
Heavy metals	<40 ppm
Mycotoxins	negative
Antibacterial activity	negative
Coliforms	<30/g
E. coli	negative in 25 g
Salmonella	negative in 25 g
Total viable count	<50 000/g

Immobilization
The immobilization system should be described in detail. Tests to indicate the physicochemical stability of both the system and its carrier and enzyme are essential.

These regulatory aspects would be acceptable for biocatalysts.

2.2 Enzyme Suppliers for Biotransformation

There are more than 400 companies dealing with enzymes all over the world and approximately 12 major producers with an increasingly distinct separation of product ranges. About 60 companies produce substantial amounts of a small range and about 400 companies produce a very limited range of industrial enzymes. Japanese enzyme producers have a special range for industrial or in-house use and contribute to 12–15% of world production. There are 24 companies which supply special enzymes for biotransformation (Table 2-1).

Table 2-1. Main enzyme suppliers for biotransformation.

Company	Country
Altus Biologics Inc.	USA
Amano Enzyme Inc.	Japan
Asahi Chemical Co.	Japan
Biocatalysts Ltd.	UK
Biozyme Labs Ltd.	UK
Calbiochem Corp.	USA, Denmark
Christian Hansen AS	DK
Diversa	USA
Fluka Chemicals Ltd.	Germany, UK
Genencor Int.	Finland, USA
Genzyme Ltd.	UK
DSM (Gist)	Holland
Meito Sangyo Co.	Japan
Merck KSA	Germany
Nagase Biochemicals	Japan
Novo Nordisk AS	Denmark
Oriental Yeast Co.	Japan
Osaka Saiken KK	Japan
Roche Diagnostics GmbH	Germany
Röhm GmbH	Germany
Shin Nihon Chem Co.	Japan
Sigma Chemical Co.	USA
ThermoGen	USA
Toyobo Co.	Japan

2.3 Origins of Enzymes

2.3.1 Microbial Enzymes

More than 90% of enzymes are produced by fermentation by microorganisms, which are used to prepare industrial and special use enzymes. Prokaryotic cells and eukaryotic cells can be easily grown in culture, and the technology of scale-up is well established on an industrial scale. Various kinds of fungi, bacteria and yeast have been screened for the production of special enzymes. Extracellular enzymes, for instance hydrolytic enzymes, are secreted into liquid and solid culture and are relatively stable in cultivation media.

Production by genetically modified organisms (GMO) is becoming popular in this field, and several kinds of GMO have been used to increase the productivity of biocatalysts. When one uses GMO enzymes for industrial use, one should know the origin of the microorganisms used and how the production method was changed. The new enzyme preparation is likely to have a different compositional spectrum of enzymes and side activities. The regulations for biocatalysts are not severe at present, but are likely to become more stringent.

2.3.2
Plant Enzymes

Some proteases, such as papain, bromelain and ficin, lipoxygenases from soy bean and white germ, and peroxidase from horseradish, are typical plant enzymes. Plant proteases are extracted and partially purified to give a powder extract. Some are supplied as digestive enzymes or neutraceutical enzymes. These have the characteristics of an SH-enzyme (thiol protease) and work in the hydrolysis of racemic esters as a protease. Lipoxygenases are only available from soybean, but the activity is not high and the regiospecificity for unsaturated fatty acids is not severe. Lipoxygenases from other plants are relatively unstable and used in-house only.

2.3.3
Animal enzymes

Porcine liver esterase (PLE), porcine pancreas lipase (PPL) and arginase are well known as biocatalysts among industrial animal enzymes. PLE catalyzes very well the hydrolysis of certain kinds of prochiral diesters and is supplied as a suspension with ammonium sulfate or in liquid form. The substrate specificity of PLE is not wide, but this is a well-investigated enzyme. PPL is very cheap and a useful biocatalyst in industry. Commercial PPL is a mixture of many kinds of pancreas enzymes, and the name pancreatin is well known as a digestive enzyme. Pregastric esterase is applied for transesterification of triglycerides. Arginase from calf liver is used to produce L-ornithine from the proteinogenic amino acid-arginene. The use of animal enzymes seems to be gradually decreasing because of disease and a variable supply. In the future, animal enzymes will no doubt be replaced by microbial enzymes of equivalent performance.

2.4
Fermentation of Enzymes

2.4.1
Liquid Fermentation

Liquid fermentation is useful for the production of enzymes as well as antibiotics. It is good for scale-up and reproduction. There are two types of enzyme produced: intracellular and extracellular enzymes. With advances in genetic engineering, *Escherichia coli* is now being used to produce enzymes. When *E. coli* is used, the enzyme is accumulated inside the cell. This method is very popular.

2.4.2
Solid Fermentation

In Japan, solid fermentation is still used to produce many kinds of enzymes including lipases, proteases and acylases. Some glycotransferases are also produced by solid fermentation. In the production of proteases, solid fermentation is often used to increase the productivity in solids. On changing to liquid fermentation, the protease is not produced and its properties change. Solid fermentation is old-fashioned and difficult to scale up because of the expensive facilities needed.

2.4.3
Extraction of Enzymes

In order to improve the extraction of enzymes, organic solvents and surfactants are sometimes used.

2.5
Extraction of Enzymes

2.5.1
Microbial Enzymes

Extraction methods depend on the fermentation conditions and the microorganism, for example, liquid or solid culture, intracellular or extracellular enzyme, laboratory or production scale preparation, etc.

Because enzymes are more soluble in buffer solution than water, enzymes in solid fermentation are extracted by stirring several times in a suitable buffer solution. The pH of the buffer solution is adjusted based on the stability and the PI of the enzyme. The solid medium is removed by filtration after extraction and the crude enzyme solution is concentrated at the next step.

Liquid fermentation produces two types of product, intracellular and extracellular enzymes. In the case of extracellular enzymes, the crude enzyme solution is collected by filtration or centrifugation of the microorganism. In the case of intracellular enzymes, collection of the microorganism and extraction of enzymes after disruption is required. On a laboratory scale, ultrasound equipment or a French press is used for disruption of microorganisms. On a large or industrial scale, a mechanical grinding mill with glass beads (for instance, a Dynamill) is used. It can treat microorganisms suspended in buffer solution at a rate of 100 L/h. Another method is enzymatic disruption of microorganism with lysozyme or YL. This method is easy to apply on an industrial scale because it does not require special apparatus. The microorganism suspended in buffer is stirred for several hours in the presence of a suitable amount of lysozyme at room temperature. During the enzymatic treatment, freezing and thawing of the microorganism is effective for disruption. In this case, lysozyme should be added before freezing the micro-

organism. The combination of mechanical and enzymatic treatment is much more effective.

After disruption of the cell wall, enzymes should be extracted with buffer solution. Sometimes enzymes are adsorbed by or adhered to the cell wall and must be extracted by adding a small amount of surfactant, such as Triton X-100. The cell wall is then filtered or centrifuged to obtain the crude enzyme solution.

Recombinant heterogeneous enzyme produced by gene-modified organisms (GMO) such as *E. coli* are sometimes obtained as inclusion bodies which form insoluble aggregates and show less original activity. In order to regenerate the activity from the inclusion body, it is dissolved in the presence of denaturing agents, usually a highly concentrated guanidinium salt and urea and reducing agents, usually thiol compounds. Protein is allowed to refold into its original active conformation after removal of the agents.

2.5.2
Plant Enzymes

Some proteases, papain and bromelain, are derived from plants and are extracted from fruits. The fruits are ground by a grinder or cutter and the proteases are extracted by buffer solution. A diluted cooled buffer is more effective than water for extraction and the extracted solution including desired enzymes should be cooled during all treatments. The content varies depending on the time and place as well as the plant.

2.5.3
Animal Enzymes

Animal organs containing desired enzymes are stored frozen and then ground and crushed by a homogenizer. Enzyme stabilizers or protease inhibitors are sometimes added on homogenizing the organs, and a buffer is preferable for extraction. In order to improve the extraction, the residue is removed by filtration or by centrifugation to obtain crude extract. Animal organs contain various kinds of enzymes and a large volume of protein is extracted. Even relatively unstable enzymes retain their activity in crude extracts, but it is necessary to purify the enzymes step by step.

2.6
Concentration

After the extraction of extracellular enzymes of solid culture or the fermentation of extracellular enzymes of liquid culture, the fermentation medium including desired enzymes is centrifuged or filtered to remove the microorganism etc. The next step is concentration by evaporation under reduced pressure or ultrafiltration (UF) to reduce the volume of the enzyme solution. It is not easy to evaporate large amounts of water under reduced pressure. Evaporation should be carried out at <30 °C except

in the case of thermostable proteins, which can be evaporated at higher temperatures. The concentrations of the salt and other soluble materials of the concentrated solution are increased by evaporation.

The most convenient and simple method for production is ultrafiltration. The method uses membrane tubes with pore sizes from about 6000 to 50000 Å. Small molecules like salt ions as well as water pass through the pores of the membrane tube while large molecules like proteins remain inside the tube. The concentration of the buffer solution is the same before and after ultrafiltration. The leaking of desired proteins in permeates should be checked during the concentration stage. Regular maintenance is carried out by using a standard protein. The membrane tube is made of polyethylene, polypropylene etc., and the irreversible adsorption of desired proteins should be avoided. The materials for the membrane should be selected before use. The flow rate of ultrafiltration depends on the facility and the protein solution applied. The final protein concentration is up to about 100 g/L.

Ultrafiltration is also used for desalting. Smaller membrane tubes are used for this purpose.

Another concentration method is precipitation using organic solvents or salts as described in Sect. 2.7.2. Ethanol is especially useful for this purpose. Despite the volume of organic solvents, it is still frequently used as a first step in the purification.

2.7 Purification of Enzymes

2.7.1 Chromatography

Chromatography is the major purification method. The choice of technique is determined by the overall yield, efficiency, speed and convenience. To purify a desired enzyme in the broth, a combination of different types of chromatography is an effective approach.

2.7.1.1 Ion Exchange Chromatography (IEX)

Ion exchange chromatography (IEX)[7] is the most typical and frequently used method for separating enzymes. Some of the advantages of IEX are high resolution power, applicability, and ease of control and scale-up. There are two types of exchanger in IEX (Figs. 2-4 and 2-5). Positively charged exchangers have negatively charged counter-ions (anions) available for exchange and are called anion exchangers. Negatively charged exchangers have positively charged counter-ions (cations) and are called cation exchangers.

The basic principle of separation in IEX is the reversible adsorption of charged protein molecules dissolved in a buffer solution by oppositely charged ion-exchanged groups on the matrix (Fig. 2-6).

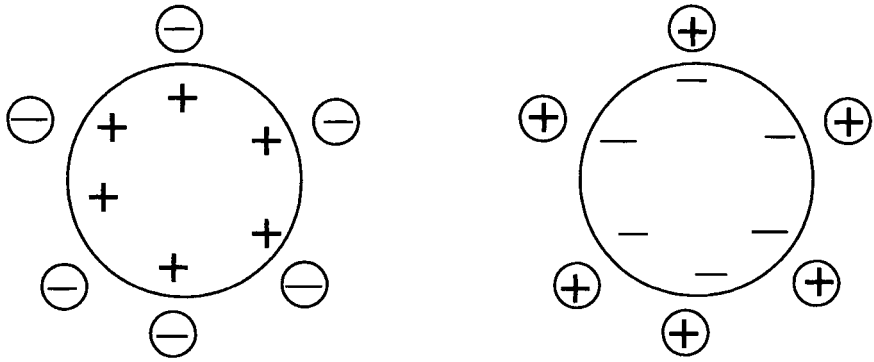


Figure 2-4. Two types of ion-exchanger[7].

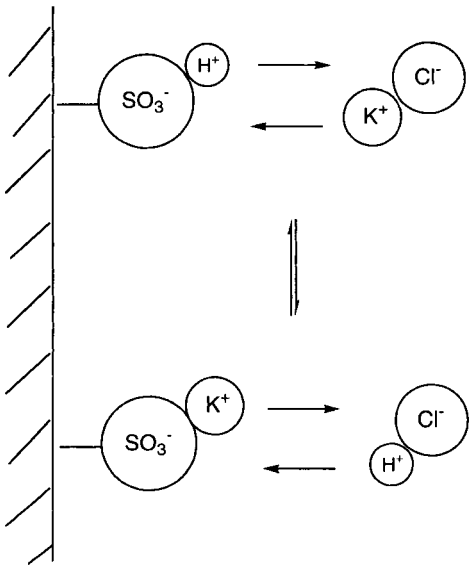


Figure 2-5. The principle of ion exchange equilibrium[4].

Proteins dissolved in buffer solution have different charges depending on the solution. When the pH of the buffer solution is below the isoelectric point (PI), the protein has a positive charge, and when the pH is above the PI, the protein has a negative charge (Figs. 2-7 and 2-8).

An ion exchanger consists of a solid matrix covalently bound to a charged group. The matrix is made of an organic compound, synthetic resin or polysaccharide, such as Sepharose and Sephadex. A typical matrix is a round microbead. The characteristics of the matrix determine its chromatographic properties, for instance, efficiency, capacity, recovery, chemical stability, mechanical strength and flow proper-

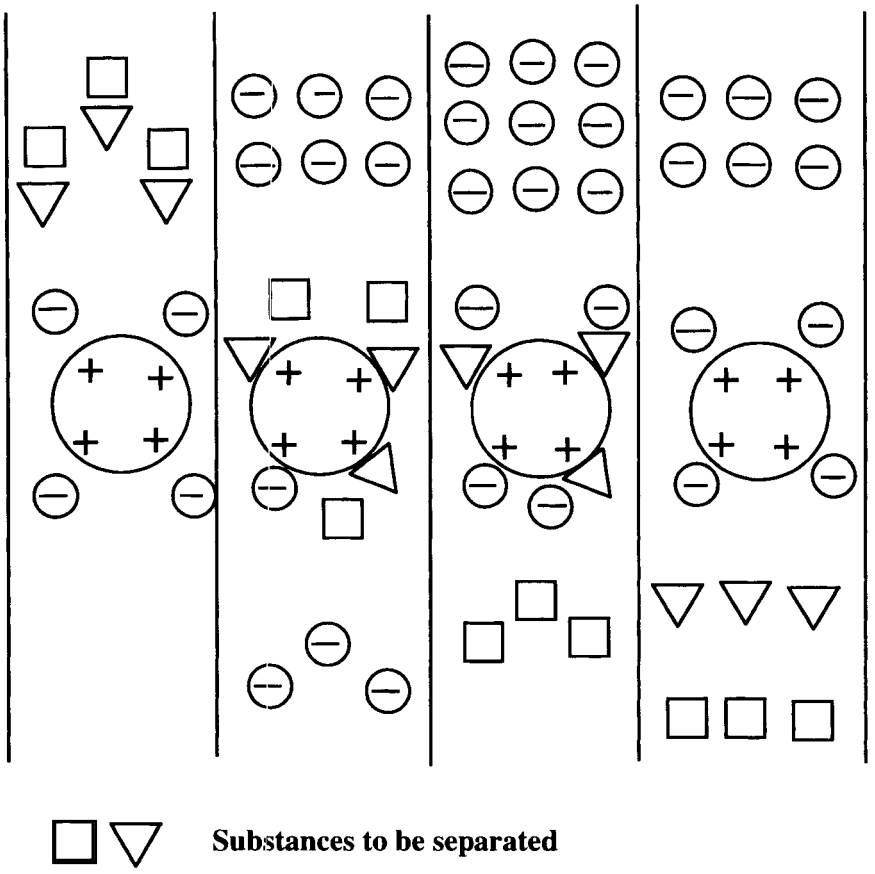


Figure 2-6. The principle of anion exchange chromatography[7].

ties. The properties of the matrix affect its behavior towards biological substances and the maintenance of biological activity.

The charged group determines the basic property of IEX, such as the type and the strength of the ion exchanger. The number of charged substituent groups per gram of dry ion exchanger or per mL of swollen gel affects its total ionic capacity. It can be measured by titration with a strong base or acid and is shown as μmol/mL gel. Typical functional groups are shown in Table 2-2 and are classified in two types. The functional groups of anion exchangers are substituted ammonium groups. Cation exchangers have sulfoxyl or carboxyl groups. Ion exchangers with sulfonic and quaternary amonium groups are called "strong ion exchangers". Those with carboxyl and diethylaminoethyl groups form "weak ion exchangers". The terms strong and weak refer to the extent of the variation of ionization with pH and not the strength of binding. A strong exchanger is ionized over a broad pH range, a weak one over a narrower range.

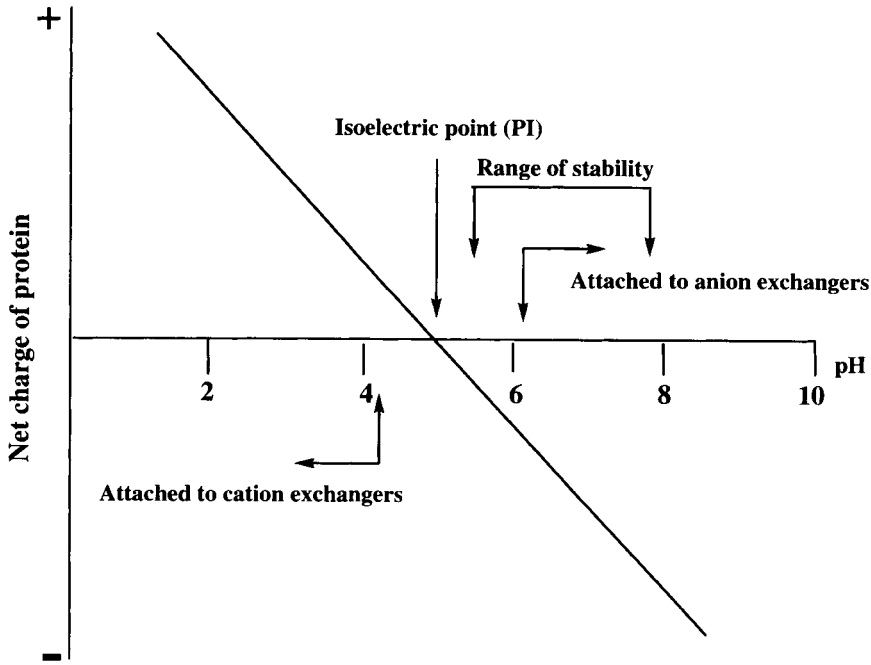


Figure 2-7. The net charge of protein as a function of pH [7].

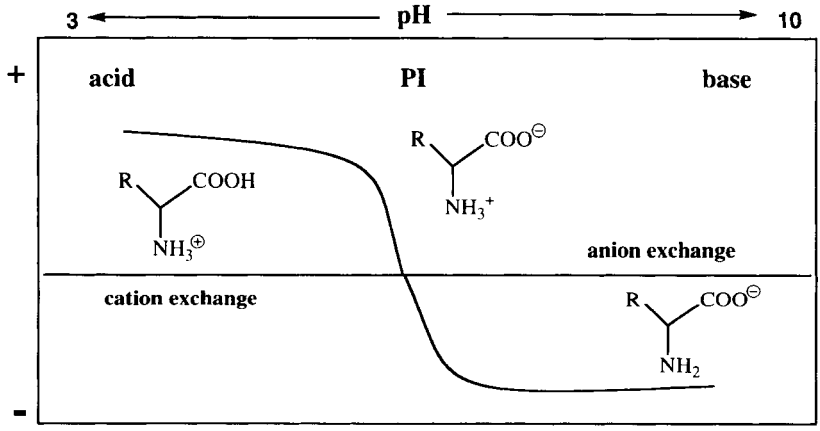


Figure 2-8. Relation between the charge of proteins and the pH [6].

Experimental Design

The choice of matrix and functional group depends on the pH stability, molecular size and isoelectric points (PI) of the protein, and on the requirements of the application. PI can be measured by electrophoresis or can be checked in the comprehensive lists of PI for proteins.

Table 2-2. Functional groups used on ion exchangers and its structure.

Ion exchangers	Structure
Anion exchangers	
Dimethylaminoethyl (DE)	$-CH_2CH_2N(CH_3)_2$
Diethylaminoethyl (DEAE)	$-CH_2CH_2N(C_2H_5)_2$
Quaternary ammonium (QA)	$-CH_2N^+(CH_3)_3$
Quaternary ammonium (QAE)	$-CH_2CH_2N^+(C_2H_5)_2CH_2CH(OH)CH_3$
Quaternary ammonium (QMA)	$-N^+(CH_3)_3$
Cation exchangers	
Carboxymethyl (CM)	$-CH_2COOH$
Phosphate (P)	$-PO_4H_2$
Sulfonic ethyl (SE)	$-CH_2CH_2SO_3H$
Sulfonic propyl (SP)	$-CH_2CH_2CH_2SO_3H$

The starting pH of buffer is chosen so that proteins to be bound to the exchanger are charged. So, the starting pH is at least more than 1 unit above the PI for anion exchangers or at least less than 1 unit below the PI for cation exchangers to facilitate adequate binding. Proteins begin to dissociate from ion exchangers at about 0.5 pH units from their PI at ionic strength 0.1 M.

Most proteins have their PI within the acidic range, so they are usually negatively charged in neutral buffer solution and show the properties of as anion.

Ion exchange separation is carried out using the following three procedures: column chromatography, a batch method and an expanded bed adsorption. Industrial- scale preparation is used.

Column Separation

Ion exchangers are available for laboratory-scale separations, and factors such as cost and reproducibility etc. are not very important. For industrial separation, however, it is necessary to optimize the purification conditions.

The DEAE exchanger is the most useful in terms of the PI and stability of most proteins.

Choice of Exchanger Group and Buffer

The choice of ion exchanger and buffer solution is limited by the stability of the proteins. Because most proteins have their PI in the acidic range, they have a slightly positive charge below the PI and can be easily absorbed on a cation exchanger (ex. CM). On the other hand, they have a negative charge above the PI and an anion exchanger (ex. DEAE) is used.

Choice of pH and Ionic Strength

The pH of the buffer depends on the PI of the proteins, and the ionic strength causes absorption on the ion exchanger and desorption from it. The required concentration of starting buffer depends on the nature of the buffering substance. It should be at

least 10 mM. Suitable ion salts stabilize the proteins in solution and excess salts cause the denaturation and precipitation of the protein.

Batch Separation

Batch separation is conducted in the same way as column development by a stepwise elution method. Batch separation is a better method for large sample volumes with low concentration protein. Large volumes take a long time. The initial conditions for batch separation are almost the same as for column chromatography, for instance, buffer pH and ionic strength etc.

It is necessary that the conditions are rather strong so that the proteins completely bind to the adsorbent. In order to increase the recovery, the pH of the buffer is kept a couple of units away from the PI of the protein. Batch separation is simple and useful to concentrate a low protein solution, but the resolution is not high.

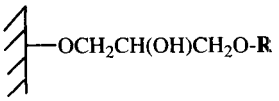
Experimental Procedure

Batch separation is a simple method whereby the protein solution is stirred together with the ion exchanger in an appropriate buffer for 1 h. The slurry is collected by filtration and the ion exchanger is washed with fresh buffer solution. When no desired protein is observed in the filtrate, a couple of bed volumes of the elution buffer are added and stirred for 1 h to desorb the desired protein. The solution including the desired protein is collected by filtration. The change of pH or ionic strength of the elution is determined by gradient or stepwise chromatography.

2.7.1.2
Hydrophobic Interaction Chromatography (HIC)

Hydrophobic interaction chromatography[8] is based on the hydrophobic properties of proteins and the hydrophobic ligands covalently bonded to the matrix. The chromatography has three forms: hydrophobic chromatography, where both absorption and desorption are based only on hydrophobic binding, hydrophobic-ionic chromatography, where absorption is based on hydrophobic binding and desorption on ionic exclusion by changing the pH of the buffer, and mixed function chromatography, based on hydrophobic, ionic and hydrogen binding.

HIC and reversed phase chromatography (RPC) are very similar principles based on hydrophobic interaction. Adsorbents for RPC are much substituted with hydrophobic ligands, such as octadecyl, octyl or phenyl groups (Figs. 2-9 and 2-10). Protein binding to RPC adsorbents is usually very strong in spite of the concentration of salt, and some kinds of organic solvents, such as acetonitrile and *iso* propyl alcohol, are used to desorb the protein. So, RPC is carried out for low molecular weight molecules such as peptides because most proteins are unstable in highly concentrated organic solvents. On the other hand, adsorbents for HIC are less substituted with similar groups, mainly butyl groups. Protein binding to HIC adsorbents requires a neutral salt like ammonium sulfate in the mobile phase, and the protein is desorbed by decreasing the concentration of salt. The slope of ionic strength in HIC is opposite to that of IEX.



R-

C_2H_5-	Ethyl
C_4H_9-	Butyl
C_6H_{13}-	Hexyl
C_8H_{17}-	Octyl
$C_{10}H_{21}$-	Decyl
C_6H_5-	Phenyl

Figure 2-9. Hydrophobic ligands attached to matrix.

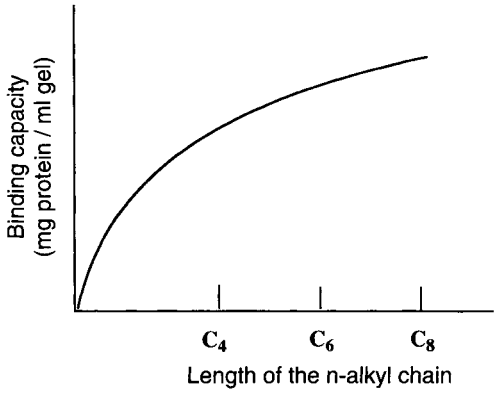


Figure 2-10. The effect of alkyl chain length and degree of substitution on binding capacity in HIC[8].

The surface of a protein is relatively hydrophilic in the lower concentration buffer solution, but hydrophobic interaction increases at high ionic strength (Fig. 2-11). It is estimated that 40–50% of the surface area of a protein is non-polar.

The HIC parameters are type of ligand, degree of substitution, concentration of salt, and effect of temperature and pH.

The immobilized ligands used are hydrocarbon groups like butyl and octyl groups and phenyl groups. The polarity of the ligand increases with alkyl chain length and its degree of substitution. The interaction of the phenyl group is not simple because of an aromatic effect as well as hydrophobicity.

The most typical salt in HIC is ammonium sulfate. As the concentration of ammonium sulfate is increased, the amount of protein adsorbed on the ligand increases linearly up to the precipitation point. The effect of the ion used in HIC the precipitation of proteins is shown in Table 2-3. Sodium, potassium or ammonium sulfates have a relatively high salting-out effect and molar surface tension of water an increasing effect.

Ammonium (1M) sulfate is a good starting point for experiments. If the protein does not retain the ligand, a more hydrophobic ligand should be selected. The recovery of protein in HIC should be >80%. When a small amount of miscible organic solvent is needed, the ligand should be changed to a less hydrophobic one.

Hydrophobic interaction in HIC is diminished by increasing the pH and increased by decreasing the pH. The PI of protein is in the acidic range and the hydrophilicity increases in the basic range. Hydrophobic interaction strength changes strongly at a pH below 5 or above 8.5. Also, on increasing the temperature of HIC, hydrophobicity slightly increases. A small amount of miscible organic solvent affects the decrease in hydrophobicity of protein and facilitates elution in the buffer solution.

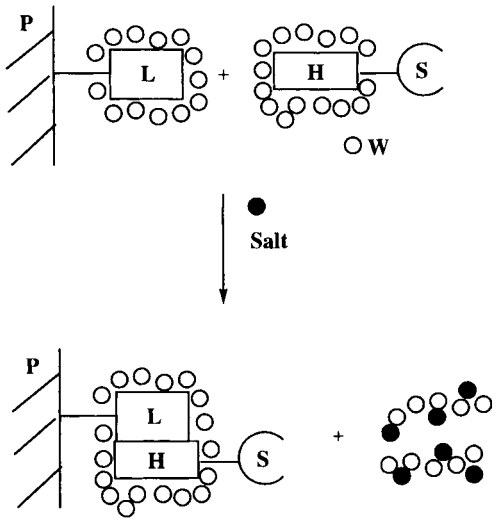


Figure 2-11. The principle of hydrophobic chromatography[8].

P: Polymer matrix
S: Soluble molecule
L: Ligand attached to polymer matrix
H: Hydrophobic patch on surface of soluble molecule
W: Water molecules in the bulk solution
S: Salt (ammonium sulfate)

Table 2-3. The effect of some anions and cations in precipitating proteins[4].

Protein	Antichaotropic effect ↔ Chaotropic effect
Ribonuclease	$SO_4^{2-} < CH_3COO^- < Cl^- < Br^- < NO_3^- < ClO_4^-$ $NH_4^+, K^+, Na^+ < Li^+ < Ca^{2+}$
Collagen Zelatin	$SO_4^{2-} < CH_3COO^- < Cl^- < Br^- < NO_3^- < ClO_4^- < I^- < SCN^-$ $NH_4^+ < Rb^+, K^+, Na^+, Cs^+ < Li^+ < Mg_2^+ < Ca^{2+} < Ba^{2+}$

2.7.1.3
Gel Filtration (GF)

Gel filtration[9] is a key method in the purification of enzymes as well as biological macromolecules. It is reliable and simple as a separation technique without adsorption and interaction on gel filtration media. In gel filtration, the principle of separation is very simple, and macromolecules in solution are separated based on differences in their size as they pass through a column (Fig. 2-12). Large molecules pass through the stationary phase first while smaller molecules move about the gel filtration medium slowly. Gel filtration is also called molecular-sieve chromatog-

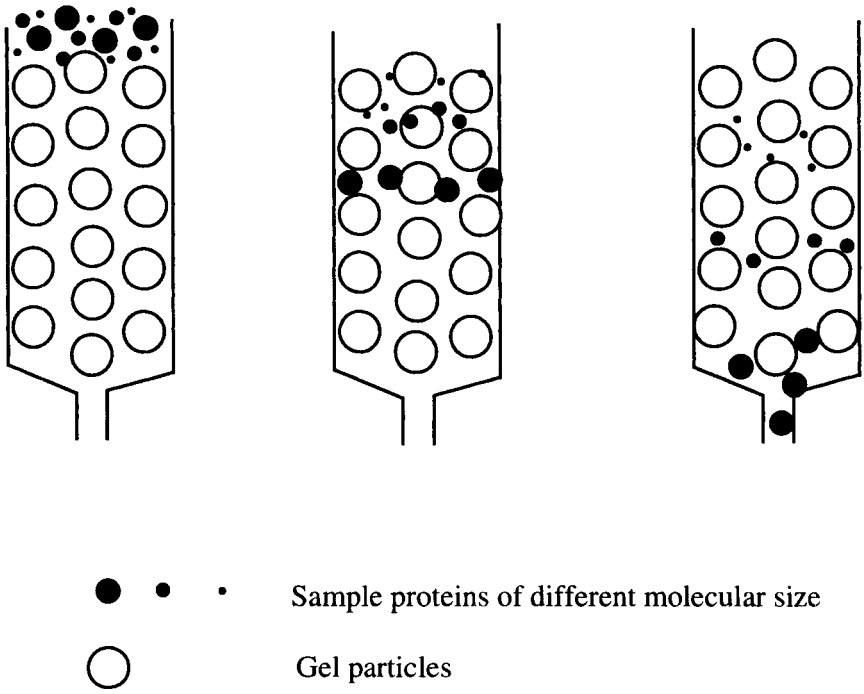


Figure 2-12. The principle of gel filtration.

raphy. Molecules are eluted in the order large to small. Gel filtration is usually used at the final or latter stage for changing buffer and concentration.

Gel filtration is carried out using a single buffer solution of appropriate pH and ionic strength. Some gel filtration media have a small number of ionic charged groups, such as carboxyl and sulfonic groups, which sometimes cause non-specific adsorption of basic proteins at low ionic strengths. In order to avoid the adsorption, gel filtration should be carried out at an ionic strength above 0.15 M. Non-ionic interactions between proteins and gel filtration media are negligible at buffer concentrations between 0.15 M and 1.5 M. An ionic strength below 0.15 M causes a slight retardation of basic proteins and exclusion of acidic proteins.

The first gel filtration medium Sephadex, provided by Pharmacia, was a bead-formed gel prepared by cross-linking dextran with epichlorohydrin. Gel filtration media with various particle size grades are now available and globular proteins can be separated between 700 and 4×10^7 Å. The fractionation range of the medium determines the porosity of the gel and is measured using typical globular biological molecules or dextrans. The shape (its diameter and length) of biological molecules affects the theoretical separation. When the molecule is not globular but a linear string, the separation is quite different.

Choice of Column, Sample Volume and Flow Rate

There are some factors affecting the choice of column equipment in order to obtain a good separation. The length of column is necessary more than 30 times the diameter of column, because the resolution increases at the square root of column length. That is why a longer column is used for gel filtration especially for an analytical fractionation. A bed length of more than 1 m is not useful and effective for industrial separation.

The dead volume at the inlet and outlet should be less than 0.1%. A sample volume of 0.5–5% of the bed volume is recommended for good resolution and depends on the gel filtration medium. The relationship between sample volume, medium and resolution is described; however, the actual sample volume should be determined by experiment. A smaller sample size is not good for resolution. Up to 30% of the total bed volume can be applied for changing the buffer and salting out.

An effective flow rate for resolution is the order of 5 mL/cm^2h and up to about 25 mL/cm^2h is allowed for industrial preparations. The length of the column and flow rate are basically in an inverse relation.

2.7.1.4
Reversed Phase Chromatography

Except for a few specific applications, reversed phase chromatography (RPC)[10] is rarely used in biological purification. RPC is commonly used for the purification of organic compounds and low molecular weight peptides. The principle of RPC is similar to that of HIC. The ligand is stronger in RPC than in HIC, and includes octadecyl, octyl, butyl and phenyl groups (Fig. 2-13). The remaining silanols are quenched with trimethylsilyl groups.

RPC medium consists of hydrophobic ligands chemically bonded to porous microbeads. The microbeads are made of silica gel or a synthetic organic polymer like polystyrene.

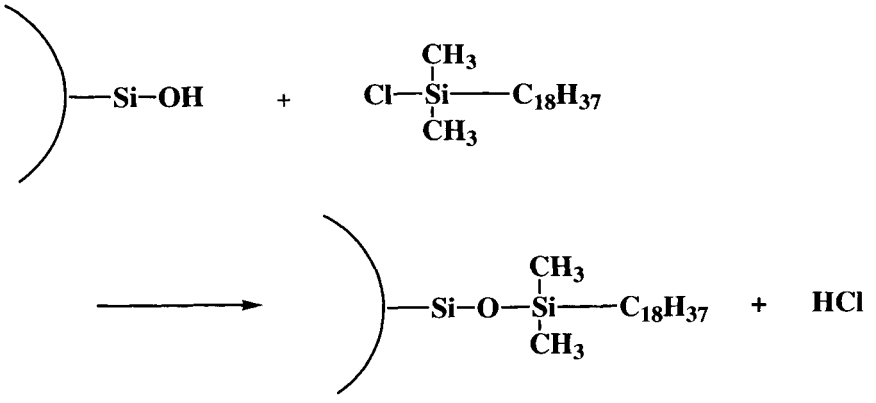


Figure 2-13. Reacting a silanol with octadecyldimethylsilyl group.

Table 2-4. Solvents used in reversed phase chromatography.

Solvent	Dielectric constant (20 °C)	Viscosity (cP at 20 °C)	bp (°C)
Acetonitrile	38.8	0.36	82
Ethanol	24.3	1.20	78
Methanol	33.6	0.60	65
Propanol	20.1	2.26	98
Isopropanol	18.3	2.30	82
Water	80.4	1.00	100

It is necessary to use water miscible organic solvents in order to elute proteins (Table 2-4). Most proteins are apt to be denatured in that case. RPC is useful for the purification of small samples or peptides and is usually carried out as high performance liquid chromatography (HPLC).

2.7.1.5
Hydrogen Bond Chromatography

There are three types of chemical interaction between the ligands and proteins: ionic, hydrophobic and hydrogen bond interaction. Hydrogen bond chromatography is not as popular as ionic and hydrophobic chromatography. Precipitation of proteins is sometimes observed in the presence of a water-soluble polysaccharide and polyethylene glycol. The complex with proteins easily forms via hydrogen bonding at high ionic strength. Ionic cellulose, such as DEAE-cellulose and CM-cellurose, as well as cellulose, is used as a matrix for this purpose. Hydrogen bond – ion chromatography is complicated because the ionic strength of the buffer solution used for each of the two methods is opposite to elute proteins.

Protein is adsorbed on DEAD-cellulose in buffer solution with 3 M ammonium sulfate and is eluted by decreasing the concentration of ammonium sulfate or adding the releasing reagents, such as urea and sucrose etc. Sodium formate and sodium acetate are used instead of ammonium sulfate. On the other hand, small amounts of ethanol, glycerol and ethylene glycol are available for elution.

2.7.1.6
Affinity Chromatography

The principle of affinity chromatography is shown in Fig. 2-14.

Affinity chromatography[11] is used for a biologically specific ligand bound to the matrix. The protein binds with ligand specifically in an active form and the rest passes through without adsorption upon washing with a buffer solution.

The ligand should have specificity and reversibility for the protein and release it on affinity elution or change of ionic strength and pH. Interactions between proteins and ligands include ionic binding, hydrogen binding and hydrophobic binding. The factors necessary for ionic binding have been listed before. The effect of ionic

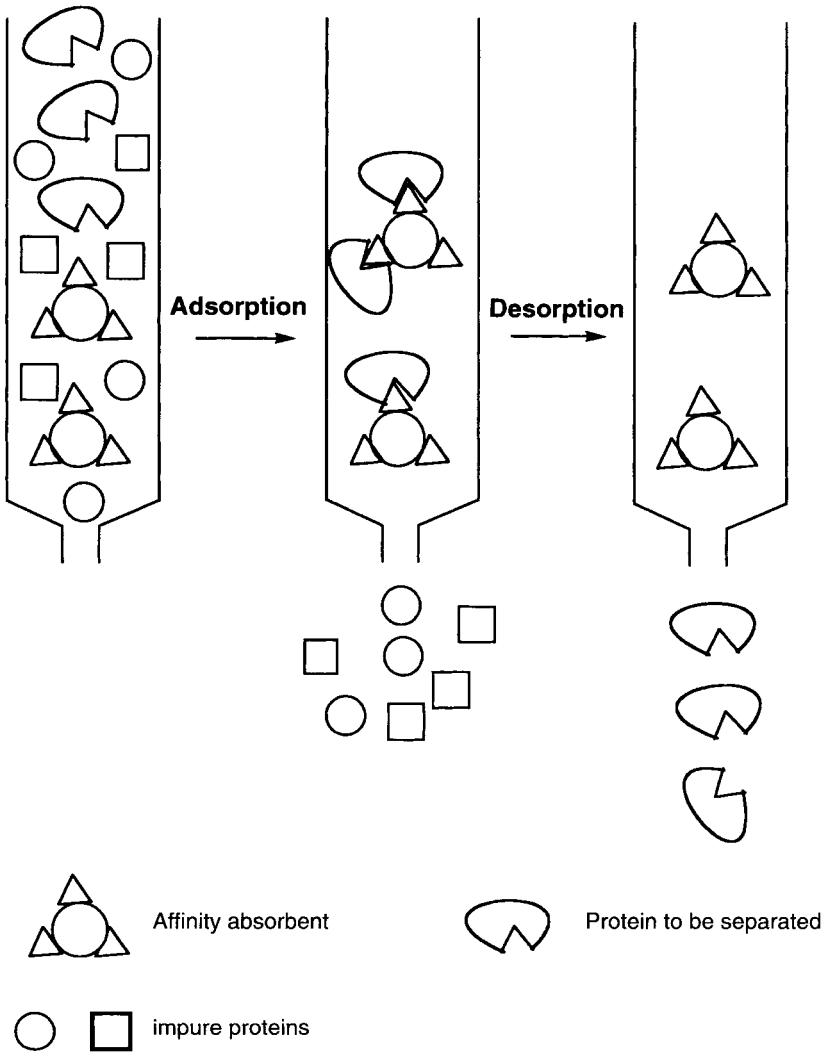


Figure 2-14. The principle of affinity chromatography.

strength on ionic binding is opposite to that on hydrophobic binding and the recovery of protein is sometimes not good. The use of a 1–3 M urea solution or 5–20 % sucrose is a good idea in such a case.

Affinity chromatography is carried out by the batch and column methods. The procedure involves (i) equilibration of the adsorbent, (ii) preparation of sample, (iii) application of the sample, (iv) washing away of unbound materials, (v) elution and (vi) regeneration of adsorbent.

When the ligand has a simple specificity for protein, about 90 % purified protein is obtained by one-step purification. So, affinity chromatography is a revolutionary

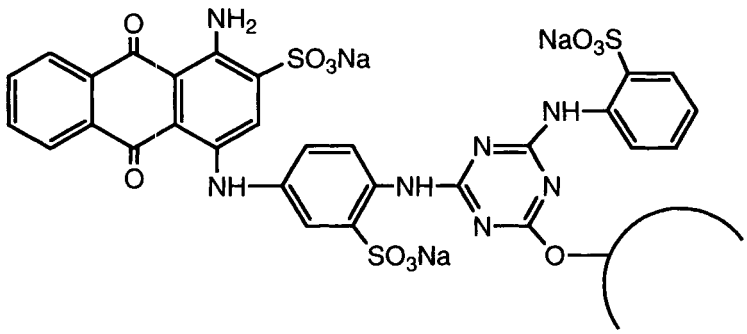


Figure 2-15. Immobilized dye chromatography [11].

purification method. Adsorbents are relatively expensive and affinity chromatography is useful for small scale purification.

There are several types of affinity chromatography. Two typical types, immobilized dye chromatography and metal chelate affinity chromatography, are described.

Immobilized Dye Chromatography

Immobilized dye chromatography is the most useful affinity chromatography. Its ligands are synthetic polycyclic dyes. These structures are very similar to the cofactors NAD^+ and $NADP^+$ as a dinucleotide analog, which are apt to bind strongly with a protein like kinases, dehydrogenases, etc. (Fig. 2-15). Some of the proteins bind biospecifically with the dye because of its structual similarity to NAD and NADP. Some proteins like lipoproteins and albumin bind in a less specific manner by electrostatic and hydrophobic interactions with the aromatic anionic ligand. Bound proteins can be eluted by affinity elution using low concentration free cofactors. On the other hand, non-specifically bound proteins need a much higher concentrate of cofactors or ionic strength.

Metal Chelate Affinity Chromatography

Metal chelate affinity chromatography is a kind of separation method which has, as a ligand, a metal ion. Some proteins and peptides are purified on the basis of affinity for metal ions immobilized by chelation on the adsorbents. Histidine and cysteine form complexes with the chelated metals around neutral pH. Biological proteins include many histidines as well as recombinant proteins as polyhistidine fusions: for instance, His-tag proteins have a specific metal chelate affinity. The adsorbent is prepared by coupling a metal chelate ligand with an iminodiacetic acid group, which forms a chelate with divalent metal ions such as Zn^{2+}, Cu^{2+}, Cd^{2+}, Hg^{2+}, Co^{2+}, Ni^{2+}, Fe^{2+}, etc.

Elution is carried out by reducing the pH and increasing the ionic strength of the buffer or by adding EDTA to the buffer. The most typical method is to gradually add sodium chloride (0.5–1.0 M) or imidazole (0–0.05 M). This ligand is very expensive, so metal chelate affinity chromatography is used only for small scale purification.

His-tag proteins produced by a recombinant are easily purified by metal chelating chromatography. His-tag proteins have about 6 histidines at the N- or C-terminal site and the His-tag easily forms a chelate with Ni^{2+}, Zn^{2+} and Cu^{2+} Elution of His-tag proteins is carried out by increasing the concentration of imidazole in the buffer solution.

2.7.1.7
Salting-out Chromatography

Salting out is popular for the purification of proteins. Salting-out chromatography is a precise method based on the same principle; however, it is not popular. Both positive and negative salting-out chromatography are carried out. The former is a combination of molecular-sieving chromatography and salting out. Proteins in buffer solution are applied to a concentration gradient column of salts. With the latter method, the precipitation of proteins salted out in the presence of celite is filled in the column and proteins are eluted with a flowing buffer solution by decreasing the concentration of salt.

2.7.2
Precipitation

Among the methods of purifying protein, precipitation is the most useful and typical for both small and large scale procedures. The precipitation methods are classified into 4 types, salting-out, organic solvent precipitation, pH changing precipitation and water-soluble precipitation. The precipitation is usually carried out early and the total protein concentration should be >0.1 mg/mL.

2.7.2.1
Precipitation by Salting out

The solubility of macromolecules like proteins in water generally increases in the presence of a suitable concentration of salt, so-called salting in. Furthermore, increasing the concentration of salt further leads to a decrease in the solubility of the proteins and their precipitation (salting out).

Salting out much depends on the pH and temperature of the solution. Proteins show minimum solubility around their isoelectric point (PI) in water and a little lower solubility in buffer solution, in other words, in the presence of salt. Regarding temperature, the solubility of proteins generally decreases at higher temperature in buffer solution with higher ionic strength.

Many salts are used for salting out, including ammonium sulfate, sodium sulfate, potassium phosphate, magnesium sulfate, sodium citrate, sodium chloride etc. The solubility of these salts is independent of temperature, and the salts do not affect the denaturation of the proteins. Ammonium sulfate is the most effective salt for salting out because of its high solubility at any temperature and low cost. Ammonium sulfate is also a useful stabilizer for proteins.

2.7.2.2
Precipitation by Organic Solvents

Water miscible organic solvents, such as ethanol, isopropanol and acetone, reduce the solubility of proteins by decreasing the dielectric constant of aqueous solution and taking away water from around the proteins. Also, these organic solvents can remove the lipids bound to a protein. Precipitation by organic solvents is affected by temperature, ionic strength and the pH of the buffer solution.

Common proteins are precipitated at about 40% in ethanol, but proteins with hydrophobic surface are soluble, like lipases, under the conditions. Alcohol concentrations of 80–90% are necessary to precipitate lipases. The concentration of protein should be >1mg/mL and that of buffer solution <50 mM. The solution and organic solvent should be cooled and the mixture kept at below 0 °C during the addition of organic solvents.

2.7.2.3
Precipitation by Changing pH

There are two types of precipitation by pH change, isoelectric point (PI) precipitation and acidic precipitation.

PI precipitation is suitable for a protein with very low solubility and is more effective in combination with salting-out and organic solvent precipitation. Anions bind with proteins more easily than cations, so the PI of proteins shifts a little to the acidic range.

On the other hand, acidic precipitation is good when the protein is stable, but impure proteins are unstable in the acidic range.

2.7.2.4
Precipitation by Water-Soluble Polymer

Precipitation by water-soluble polymer is a simple method for the purification and crystallization of proteins. Many proteins are easily precipitated in the presence of water-soluble non-ionic polymers such as polyethylene glycols (PEG 2000, 4000, 6000), methyl cellulose, polyvinyl alcohol (PVA) and dextrans (DEX).

These water-soluble polymers take away water from around proteins. The proteins bind with these polymers via hydrogen bonds, and then the complex precipitates as a solid or sometimes becomes a viscous liquid. Hydrogen bond chromatography is based on this principle.

The complex contains water-soluble polymers, which must be removed by ionic chromatography, salting out, ethanol precipitation, electrophoresis etc.

2.7.3
Crystallization

Relatively purified proteins are easily crystallized at >1%, usually 5–10%, of the protein concentration in buffer. So, crystallization is the final stage of purification, and useful for storage of proteins and X-ray crystal structure analysis. In protein chemistry, crystallization does not mean the protein is 100% pure even though it is in crystalline form. As described for salting out, a crystallized protein is in a solid state together with precipitation aids such as salts, organic solvents, water-soluble polymers etc.

Freeze drying is one of the crystallization methods; however, denaturation, deactivation, or a slight change in the three-dimensional structure of a protein is sometimes observed. It is necessary to check the stability before freeze drying.

2.7.4
Stabilization During Purification

Care must be taken not to lose the activity during purification of the enzyme after fermentation. Enzymes are affective macromolecules influenced by changing the pH, temperature, the concentration of buffer and salts, metal ions, detergents, organic solvents and so on. In order to preserve their activity, enzymes should be kept under natural physiological conditions such as a low temperature of about 4 °C, natural pH for the enzyme, physiological buffer solution and concentration, etc. Some additives for enzyme stabilization are used during purification. Mercaptoethanol and dithiothreitol work as antioxidants and EDTA works as a chelating agent to prevent inactivation by heavy metal ions and metalloproeases.

Polysaccharides like dextrin, sugars, sugar-alcohols like sorbitol and mannitol, glycerol and ethylene glycol are sometimes used as stabilizers. Some peptides and amino acids are useful excipients for purification. Compounds with a similar structure to that of the substrate are generally effective as stabilizers and are used as fillers for storage.

Degradation by proteases derived from the same microorganism or from contamination during purification must be avoided. Once a protease contaminates an enzyme solution, the desired enzyme is degraded during purification and might disappear. To prevent degradation by proteases, it is helpful to add protease inhibitors like PMSF (SH protease) and EDTA (metal protease).

2.7.5
Storage of Enzymes

2.7.5.1
Storage in Liquids

Common enzymes in liquid form should be stored below 4 °C in a refrigerator and kept with a stabilizer. Most enzymes keep their activity for several years under suitable conditions, especially thermostable enzymes.

Ammonium sulfate (2 M) is a popular storage solution for commercial porcine liver esterase (PLE). Ammonium sulfate prevents microbial growth on the solution. Storage in 50% glycerol is also useful and this glycerol stock can be stored below 0 °C.

2.7.5.2
Storage in Solids

Solid forms for storage are preferred in commercial enzymes. Generally, an enzyme is much more stable in solid form than in liquid form even without a stabilizer. A solid form for storage is prepared by precipitation with organic solvents and freeze drying or spray drying depending on the purification stage. Precipitation using an organic solvent is convenient, but the purity is not so high. Freeze drying is very useful but expensive. Spray drying is preferable for commercial enzymes. Spray drying is commonly carried out at about 140–70 °C for which the enzyme needs moderate thermostability.

Stabilizers are effective to avoid loss of activity and the typical stabilizers described above are used during precipitation and crystallization.

Some enzymes in solid form are very stable and can be stored at room temperature for several years without loss of activity.

2.8
Commercial Biocatalysts

Among biocatalysts, hydrolases like lipases and proteases are the most popular. There are several types of biocatalysts in commercial products. Immobilized lipases and cross-linking enzymes are briefly described in this section.

The most popular immobilization method is adsorption on a carrier such as diatomaceous earth or a synthetic polymer. The advantage of this method is that the original activity of the enzyme is maintained, but the disadvantage is that the enzyme cannot be used in an aqueous solution.

Lipases immobilized on ceramics modified with a chemical silyl reagent adsorb strongly and can be used in aqueous solutions as well as organic solvents. The activity is sometimes ten times the original and the thermostability is increased. These products can be reused more than ten times depending on conditions.

Cross-linked enzymes are commercial biocatalysts and can be reused in organic solvent and aqueous solution. They are purchased as crystals derived from a single cross-linked enzyme.

Some screening kits are provided for user convenience. Main suppliers are listed in Chap. 20.

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3
Rational Design of Functional Proteins

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3.1
Protein Engineering

One of the ultimate goals of protein engineering[1] is the ability to design and synthesize a biocatalyst that meets the demand of any user. The enzyme would have to satisfy the desired activity, stability, specificity and so on in each individual case. Unfortunately at the present time, although we have the means to synthesize proteins of any desired primary structure, we are still a long way away from the *de novo* design and synthesis of enzymes. We will need to understand the structure-function correlation of proteins, and the principles of protein folding and interaction much better. Even a protein of average size will contain thousands of atoms, and therefore the number of possible inter-atomic interactions will be in the millions, and the number of conformations accessible to a protein grows exponentially with chain length. Although *ab initio* structure prediction methods (predicting three-dimensional protein structures from amino acid sequences alone) are steadily advancing, accurate predictions are still fairly limited to small proteins or structural domains.

A completely contradictory approach that has been, and is still now a major method to obtain an ideal biocatalyst, is to simply find it. This strategy leaves most of the work to nature. Adaptation of a wide variety of organisms to diverse environments on our planet has led to a massive collection of enzymes from which we can select. As the number of organisms identified keeps growing, so does the number of constituent enzymes. In particular, the recent studies on extremophiles (thermophiles, halophiles etc.), have significantly broadened the range of available biocatalysts[2]. Hyperthermophiles, which grow at temperatures above 90 C, provide a complete set of thermostable proteins that are sufficient to maintain life at these temperatures[3]. However, this approach does have its limitations. Enzyme activities towards substrates not found in nature, or properties that are not required for life such as protein stability against organic solvents, may be difficult to find.

The two methods mentioned above are the extremes, and many methods that combine the two are now being developed, or have already been applied. One

popular method is the random mutagenesis of a gene encoding a particular protein of interest, for example a lipase. A lipase that most satisfies the demands would be chosen rationally, to try to optimize the enzyme. Random mutagenesis, or recently, DNA shuffling[4], of the lipase gene produces a collection of proteins that resemble the original lipase from which we can select. If the structure of the lipase is available, it may be possible to define the region that should be subject to mutagenesis rationally, thereby raising the fraction of improved mutants. The structure may also allow us to pinpoint rationally one or more particular residues as targets for site-directed mutagenesis. This is usually followed by biochemical and structural evaluation of the variant protein, comparison with the wild-type protein, interpretation of the data, and then the designing of a further modification. This will have to be repeated until the desired changes in a protein are obtained. When affinity to a particular molecule can be used as a means for selection, phage display[5], cell surface engineering[6], catalytic antibodies[7], the two-hybrid system[8], Profusion technology[9], and so on provide powerful tools for selection of a desired peptide from a *de novo* synthesized library. The simplicity of selection, which in many cases is basically the binding of the peptide to a molecule immobilized on a matrix, allows multiple cycles of mutagenesis and selection in a relatively short time. Although this methodology had been considered an advantage enjoyed mainly by affinity screening, recent high-throughput technologies have enabled rapid analyses of tens of thousands of clones for various enzyme parameters such as stability and substrate specificity. Thus it is now possible to improve various parameters of biocatalysts by this methodology, called directed evolution[10–14], which is presented in Chap. 4.

In the present chapter, we will focus on the more rational approaches of enzyme engineering and design. Basic techniques for site-directed mutagenesis, protein crystallization, and comparative modeling will also be introduced. Some recent, key examples of rational protein engineering will be described in a somewhat detailed manner. There are also very informative reviews in the literature[11, 15–17].

3.2
Gene Manipulation Techniques in Enzyme Modification

The repertoire of recombinant gene technology allows us to manipulate foreign or heterologous genes in a genetically well understood organism. There may be a few exceptions, but from a general viewpoint, *Escherichia coli* is the organism of choice for protein engineering. Thanks to the general availability of easy to use cloning kits tailor-made for mutagenesis, straightforward experiments can be carried out. Even if other organisms are superior for the production of a particular enzyme, mutagenesis procedures will be carried out in *E. coli*. A variety of multi-purpose plasmids for mutagenesis and expression are readily available from commercial sources. PCR-based methodologies now make it possible to incorporate, clone, isolate and confirm gene mutations within a couple of days. *E. coli* host cells have also been dramatically improved, and an abundant collection of strains are now commercially available for various needs. Some typical techniques will be described here.

Many recombinant gene expression systems have been developed in the past years. Synthesis of the protein is controlled at the transcriptional level as in the well-known *lac* and *tac* systems[18]. Chemicals such as isopropyl-β-D-thiogalactopyranoside (IPTG) in the *lac* and *tac* systems are used to initiate and induce high levels of transcription. Although expression of the genes is low when an inducer chemical is not present, this basal expression may inhibit experiments when the target proteins are lethal to the host cell. Some systems overcome this problem by controlling gene expression under the control of a T7 promoter[18]. This promoter is specifically recognized by T7 polymerase, whose gene is introduced into the host cell. Expression of the T7 polymerase gene is regulated by an upstream *lac* promoter. When IPTG is not present, very little T7 polymerase is produced, consequently leading to minimal expression of the target gene. A *lac* operator can be inserted in between the T7 promoter and the target gene in order to achieve higher stringency. Furthermore, the gene encoding T7 lysozyme, a natural inhibitor of T7 polymerase, can also be introduced in the host cells to reduce target gene transcription under uninduced conditions further[18].

One of the major problems one might encounter when expressing foreign genes in *E. coli* is the formation of inclusion bodies when the proteins produced are host-lethal, or mis-folded. This will require the unfolding of the protein with various detergents or denaturants, followed by refolding experiments. Another problem often seen is the low levels of target gene expression in the host cells when these genes contain many codons that are not frequently used in *E. coli*. This is due to the depletion of rare tRNA species in the host cells. There are now commercially available host cells transformed with extra copies of *argU*, *ileY* and *leuW* tRNA genes to allow high-level expression of genes with rare codons. Many other strategies, such as inactivating the Lon protease gene in the host cell[18], have been applied in order to maximize the production of diverse recombinant proteins that may be of interest.

Site-directed mutagenesis methodology has also seen many advances in the recent years. Most strategies are described in detail in reference 18. In essence they all rely on synthetic oligonucleotides which contain the desired information for a modified protein sequence, be it replacement, insertion or deletion of amino acids. Classical cassette mutagenesis techniques are available, along with newly developed strategies utilizing PCR techniques.

In cassette mutagenesis, synthetic complementary oligonucleotides including the modified sequence are hybridized to form a double-stranded DNA fragment. This fragment should span a region including two appropriate restriction enzyme sites on opposite sides of the mutation. It is then easily possible to exchange the native sequence with the modified sequence after restriction enzyme digestion and ligation. When only a particular mutation is required, PCR-based methods should be less tedious and faster. However, when mutations span a relatively long region, or require completely different nucleotide sequences compared with the original gene, or when random sequences are to be introduced into a particular region, this classical method still has its advantages.

To introduce mutations into genes via PCR four instead of the normal two primers are needed. The so-called outer primers bind at the beginning and the end of the

target region, while the inner primers bind to the site where mutations are desired and consist of the modified base information. To obtain the complete sequence with the mutation, two rounds of PCR must be performed. The first reaction amplifies the DNA fragment from the beginning to the mutated region, the second one from the mutation to the end. Both PCR products carry the mutation either at the beginning or at the end of the DNA fragment. After purification of both PCR products the two amplificates are mixed, denatured and then the PCR will be started (without primers). To amplify the whole sequence with the mutation the outer primers are added and the PCR is started again. During the PCR both the mutated strand and the natural strand are amplified but after only a few cycles will there be much more DNA harboring the mutation.

There are now also methods that can efficiently introduce mutations in a single PCR run (Fig. 3-1). The procedure utilizes a double-stranded DNA plasmid with the target gene isolated from a *dam*$^+$ *E. coli* strain. Two complementary oligonucleotides with the desired nucleotide substitutions are used for PCR along with the plasmid as a template. The product is a mutated plasmid with staggered nicks. This is treated with *Dpn*I, an endonuclease specific for methylated and hemimethylated DNA. As *dam*$^+$ strains methylate plasmid DNA, the parental strain harboring the original gene will be susceptible to *Dpn*I treatment and digested, markedly enhancing the efficiency of mutant gene isolation.

The interesting part begins when comparing the original enzyme with the mutant enzyme. Thus it is advisable to run the experiments in parallel. To interpret the results various options have to be considered. Either the enzyme activity is unchanged (a so-called silent mutation) or it is changed for the better or worse. The interpretation of these changes poses a serious problem. First it has to be asked whether the mutation has altered the overall enzyme topology or whether it influenced only the local geometry. Thus besides the usual kinetic analysis some structural determination is advisable. To date X-ray crystallography and NMR spectroscopy have given the most detailed picture, CD or IR spectroscopy are of less value.

3.3 Protein Crystallization

The three-dimensional structure of a protein is the most powerful basis from which a rational approach can be taken to modify a protein. When the structure of a highly homologous protein has been determined, one may attempt to obtain structural information by comparative modeling, or homology modeling. However, the reliability of a model is questionable when similarities of the compared proteins are not high, and we are almost helpless when a structurally novel protein is the one of interest. Although rapid progress is being made in the use of NMR spectroscopy, the orthodox methodology in elucidating a protein structure is still protein crystallization and X-ray diffraction. As detailed explanations of both methodologies appear in the literature[19], we will just touch on some points concerning protein crystallization.

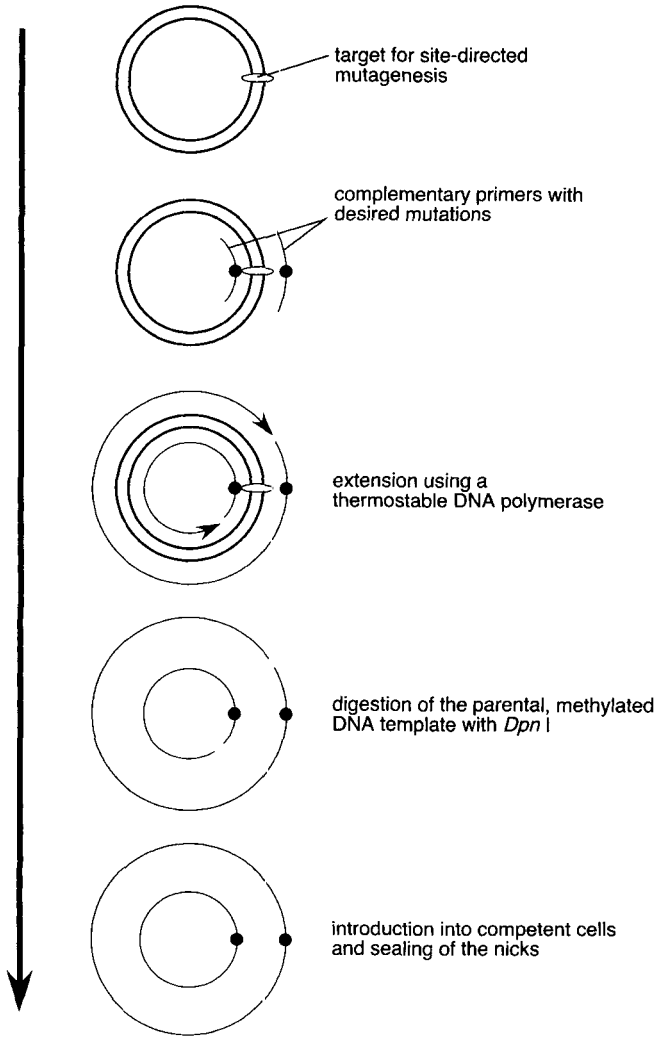


Figure 3-1. A rapid method to introduce mutations into a target gene. Thick lines represent the methylated plasmid DNA harboring the wild-type target gene.

Although there exists a vast number of protein structures in the databases, there is as yet no rational procedure to crystallize a particular protein. The procedure is still mainly based on a trial and error approach. The crystallization process itself is one of which the protein is slowly and orderly precipitated from a solution. As a general rule, the purity of the protein is the most important factor to be dealt with before attempting to crystallize a protein. If possible, care should be taken not only to remove contaminant proteins, but also to remove any structurally heterologous populations in the purified protein sample. This may be achieved by discarding tail

fractions after chromatography. Ideally, all molecules of the protein should have identical surface properties, especially in terms of charge distribution, as this will influence the packing of the molecules in the crystal. SDS-PAGE (sodium dodecyl sulfate-polyacrylamide electrophoresis), often used to display the homogeneity of a purified protein in biochemical studies, will not always provide sufficient information. Mass spectrometry can be recommended for a detailed examination of protein homogeneity.

After the purity of a protein sample is confirmed, an appropriate solvent and precipitant must be chosen. Again, there are no rational means in deciding these components. Solvents are usually a water-buffer solution, and detergents or organic solvents may be added when necessary, such as in the cases of membrane proteins or lipases with a relatively hydrophobic surface. Typical precipitants are polyethylene glycol (PEG), ammonium sulfate, sodium or lithium chloride salts, or 2-methyl-2,4-pentanediol (MPD). As multiple parameters must be considered, the search for an optimal crystallization condition may be complicated and tedious. Alternatively, convenient kits with an array of ready-to-use solutions including various buffers and precipitants are commercially available. One may screen for an appropriate crystallization condition with these kits, and then optimize the conditions based on the results.

Various techniques can be applied to crystallize proteins. Vapor diffusion using the hanging drop method is one of the popular ways to obtain crystals. In this method, a sample solution of 2–5 μL of protein solution is placed on a siliconized microscope cover glass. The same volume of precipitant solution is mixed, forming a small drop on the glass surface. The glass is then placed on a well, with the drop hanging down from the glass. Prior to this, 1 mL of the precipitant solution is poured into the bottom of the well, so that the surface does not make contact with the hanging drop. Vaseline or grease should be applied to the rims of the wells, so that an air-tight chamber is made when positioning the cover glass. In this example, the concentration of precipitant in the well is twice that of the drop. Equilibrium is reached by vapor diffusion, and the precipitant concentration in the hanging drop will gradually increase, possibly leading to crystallization. The sitting drop method can also be applied when there is a surface separated from the precipitant solution in the well. Drops are placed on the surface, and the chamber is sealed. Other methods include batch crystallization, liquid-liquid diffusion, and dialysis. Approximately 20 μg of protein are used in a single screen, therefore 50 to 100 tests will require roughly 1 to 2 mg of purified protein. In Fig. 3-2, some examples of protein crystals[20–25] are shown. Figure 3-2 A and B provide a good example of different crystallization conditions of a single protein, archaeal O^6-methylguanine-DNA methyltransferase, leading to distinct forms of crystals[20, 21]. Crystals of archaeal DNA polymerase[22, 23] and archaeal Rubisco[24, 25] are shown in Fig. 3-2 C and D, respectively.

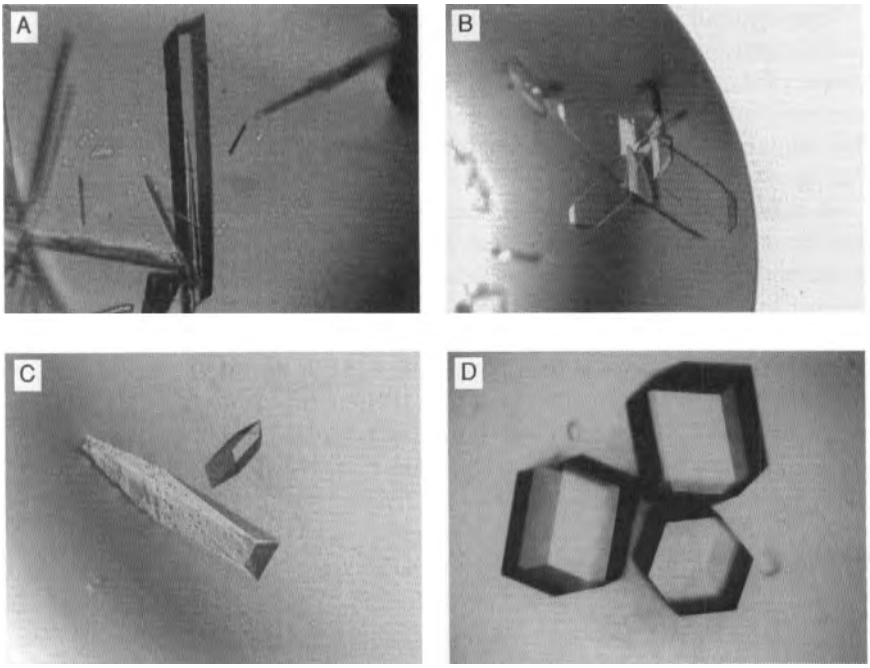


Figure 3-2. Crystals of various proteins from the hyperthermophilic archaeon, *Thermococcus kodakaraensis* KOD1. A, rod-like crystal of O^6-methylguanine-DNA methyltransferase (MGMT); B, plate-like crystal of MGMT; C, bar-like crystal of DNA polymerase; D, cubic or hexagonal crystals of ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco).

3.4 Comparative Modeling of a Protein Structure

Comparative modeling, or homology modeling, is the most powerful tool when a rational approach is taken to engineer a protein with an unknown three-dimensional structure (the target protein). Through comparative modeling, the three-dimensional structure of the target protein can be predicted based on its alignment to one or more proteins of known structure (the templates). The rapid accumulation of known protein structures and the advances in modeling software have significantly increased the accuracy of comparative modeling. It is now possible to model, with sufficient accuracy, significant parts of one third of all known protein sequences. Detailed and informative reviews[26–30] can be found in the literature, and we will only present a general overview in this chapter.

In order to predict a structural model, similarity between the primary sequences of the target and template(s) must be detectable. Furthermore, an accurate alignment of the two or more sequences must be calculated. If these requirements are met, one may proceed to model the target. The process of comparative modeling can be divided into four steps: (i) fold assignment and template selection, (ii) target-template alignment, (iii) model building, and (iv) model evaluation.

Templates can be selected using the target sequence as a query for searching protein structure databases [e.g. Brookhaven Protein Data Bank (PDB): http://www.rcsb.org/pdb/index.html; Structural Classification of Proteins (SCOP): scop.mrc-lmb.cam.ac.uk/scop/; DALI: www2.ebi.ac.uk/dali/; Class, Architecture, Topology and Homologous superfamily classification at CATH: www.biochem.ucl.ac.uk/bsm/cath/).

Methods for protein comparison can be divided into three types. BLAST and FASTA represent the first type, where the target sequence is independently compared with each sequence in the databases, using pairwise sequence-sequence comparison. The second type is represented by PSI-BLAST, which expands the set of homologs of the target sequence. In a PSI-BLAST search, an initial set of homologs against the target sequence is collected, aligned with the target sequence, and a position-specific scoring matrix is constructed from the alignment. This matrix is then used to carry out another search for new homologs, and this is subsequently repeated until no new homologs are identified. It has been reported that PSI-BLAST identifies homologs of known structure for approximately twice as many sequences than a BLAST search. The third type of search is the 3D template matching method. The target sequence is threaded through a library of known three-dimensional protein folds, and a structure-dependent scoring function predicts the suitability between the protein and the fold. This method is useful when homologs of the target sequence cannot be found in terms of primary structure comparison. After a collection of candidate templates is obtained, one should take into account the relationship of each template to the target, the quality of the templates, and other factors (e.g. the presence of convenient protein-ligand structures) before choosing the template(s) to be used for alignment and modeling. Comparisons of the relationships between protein sequences can be determined by constructing a phylogenetic tree among the candidates [CLUSTALW at European Bioinformatics Institute (EBI): http://www.ebi.ac.uk/clustalw/ or DNA Data Bank of Japan (DDBJ): http://www.ddbj.nig.ac.jp/htmls/E-mail/clustalw-j.html]. The CLUSTAL programs can be further used for target-template sequence alignment. When protein sequences display over 40% identity, the alignment is usually correct. When sequence identity is below 20%, multiple template structures should be used in order to identify specific regions or secondary structures that can be used as "guides" to construct an accurate alignment.

Once a target-template sequence alignment has been constructed, a variety of methods and software is available for model building. Modeling methods are based on rigid-body assembly, by segment matching or coordinate reconstruction, or by satisfaction of spatial constraints. We will not introduce the details of each method, and readers should refer to the indicated literature. When used optimally, all three methods usually give similar results. Furthermore, the accuracy of the alignment used in modeling is crucial, as no current comparative modeling method can compensate for an incorrect alignment. On the other hand, the evaluation of a model is usually more reliable than the evaluation of an alignment. Therefore, when a choice among candidate alignments is difficult, one should generate models from each alignment, and choose the most promising one by evaluating the integrity of

the three-dimensional models. Information concerning modeling programs such as Insight II and QUANTA are available at http://www.accelrys.com, SYBYL/Base at www.tripos.com, and Internal Coordinate Mechanics (ICM) software at http://www.molsoft.com. A detailed list of databases and software programs is shown in reference[26]. Various programs are available to the public at the ExPASy Molecular Biology Server (http://www.expasy.ch/).

After generating a structure model, it is essential to evaluate its accuracy before one can use it for rational purposes. A wide variety of programs and servers exist that can be used to evaluate a model. The BIOTECH (biotech.embl-ebi.ac.uk:8400/), ERRAT and VERIFY3D (http://www.doe-mbi.ucla.edu/Services/), and PROVE (http://www.ucmb.ulb.ac.be/UCMB/PROVE/) are servers available to the public. Model evaluation programs check various features of the model, including bond length, bond angles, main-chain and side-chain torsion angles, peptide bond and side-chain ring planarities, chirality, and clashes between non-bonded pairs of atoms. After the evaluation step, the generated structure model is ready for use as a basis for site-directed mutagenesis. The information obtained from the biochemical evaluation of variant proteins will also contribute to improvements in the model for the rational design of mutants.

3.5 What is Needed to Take a Rational Approach?

It is quite obvious that the more information that is available on a particular protein, the easier it is to take a rational approach to improving its performance. Biochemical analyses of a protein provides valuable information in terms of its activity, specificity, and stability under various conditions. Kinetic analysis of the enzyme reveals the kinetic mechanism of the reaction, in other words the order in which substrates enter and products are released from the enzyme. This gives us an idea as to what types of intermediates or complexes may be formed during the reaction. Cloning and sequencing of the genes provides the primary structure of the protein. The number of sequences available in public databases (e.g. GenBank/EMBL/DDBJ for genes, SwissProt and PIR for proteins) is enormous, and readily available (Entrez protein or nucleotide sequence search at NCBI; http://www.ncbi.nlm.nih.gov/entrez/query.fcgi). Comparative analysis of these sequences, along with their biochemical properties, may in some cases provide enough information to modify a protein rationally through site-directed mutagenesis. Furthermore, there are now an ever increasing number of three-dimensional structures of enzymes in the databases, and these provide us with the precise architecture of various enzymes. Along with advances in protein crystallization methods and X-ray diffraction technology, rapid progress has also been made in solving protein structures by alternative tools, such as NMR spectroscopy. Using the comparative modeling mentioned above, it is also possible to predict the three-dimensional structure of a protein using the determined structure of a closely related protein. Modeling software can also calculate and predict *in silico* the local structural changes of a protein after site-directed mutagene-

sis, opening the way for rational protein design. In the following sections, we will introduce some successful examples of rational improvement or alteration of enzyme biocatalysts based on various degrees of available information.

3.6 Examples of Protein Engineering

3.6.1 Protein Engineering Studies: Providing a Rational Explanation for Enzyme Specificity

Tyrosyl-tRNA-synthetase from *Bacillus stearothermophilus* is an enzyme (MW = $2 \times 47\,500$ Da) that as a dimer catalyzes the aminoacylation of $tRNA^{Tyr}$ with tyrosine in the following two steps.

$$E + Tyr + ATP = E.Tyr\text{-}AMP + PPi \quad (1)$$
$$E.Tyr\text{-}AMP + tRNA = Tyr\text{-}tRNA + AMP \quad (2)$$

This enzyme was one of the first and best case studies of protein engineering. Based on the X-ray structure, there are eleven hydrogen bond contacts between the protein and its reaction intermediate tyrosyl-adenylate[31]. Of these, eight are hydrogen bonds involving amino acid side chains, the remaining three are with backbone C = O or N - H. A great deal of detailed information about the performance of these contacts has been obtained. Thus the amino acids Tyr 34, Cys 35, Gly 36, Asp 38, His 48, Thr 51, Tyr 169, Gly 192 and Gln 195 are the relevant hydrogen bond partners for the enzyme-bound intermediate (Fig. 3-3).

The contributions that these individual hydrogen bonds can make have been rationalized[32] (Table 3-1). Thus, deletion of a hydrogen bond donor or acceptor weakens the substrate-binding energy by 0.5–1.5 kcal mol^{-1}. Deletion of the charged Asp 38 that binds to the substrate lowers the binding energy by ~4 kcal mol^{-1}. Based on Michaelis-Menten kinetics, this amounts to the following barrier:

$\Delta G^{\ddagger}_{T} = RT(k_B T/h) - RT\ln(k_{cat}/K_M)$
k_B = Boltzmann constant
h = Planck's constant
and $\Delta\Delta G$ for the mutant enzyme (mut) compared with the wild-type enzyme (wt):
$\Delta\Delta G^{\ddagger}_{T} = RT\ln\{(k_{cat}/K_M)_{mut}/(k_{cat}/K_M)_{wt}\}$

Hydrogen bonding energies in the range of 0.05–1.5 kcal mol^{-1} give discrimination rates in k_{cat}/K_M from 2-fold to 12-fold whilst 4 kcal mol^{-1} amounts to 1000-fold discriminations. The latter point explains nicely the enormous specificity for tyrosine as compared with phenylalanine. Asp 176 in the binding pocket binds to the phenolic hydroxyl group of tyrosine (Fig. 3-3). Thus from the large amount of data presented we can learn about the binding energy of hydrogen bond donors and acceptors in proteins: deletion of a side chain between the enzyme and substrate to leave an unpaired, uncharged, hydrogen donor and acceptor weakens the binding energy by only 0.5–1.5 kcal mol^{-1}. However, the presence of an unpaired and

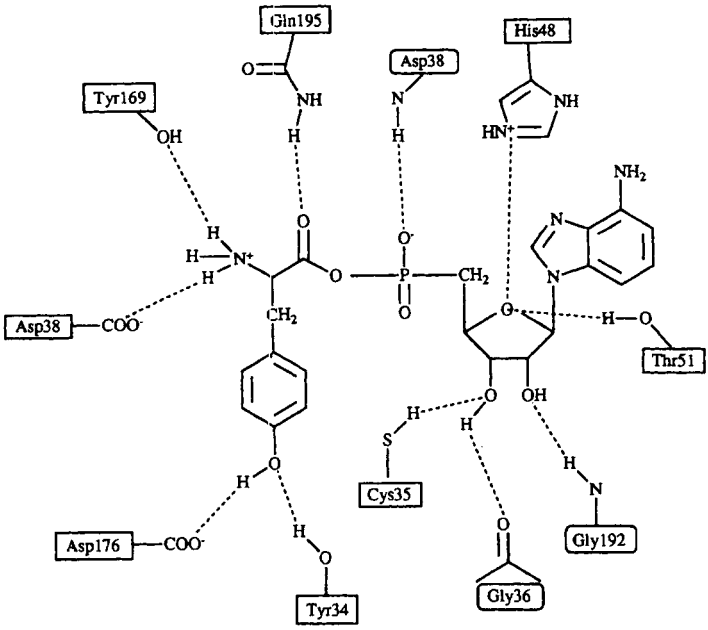


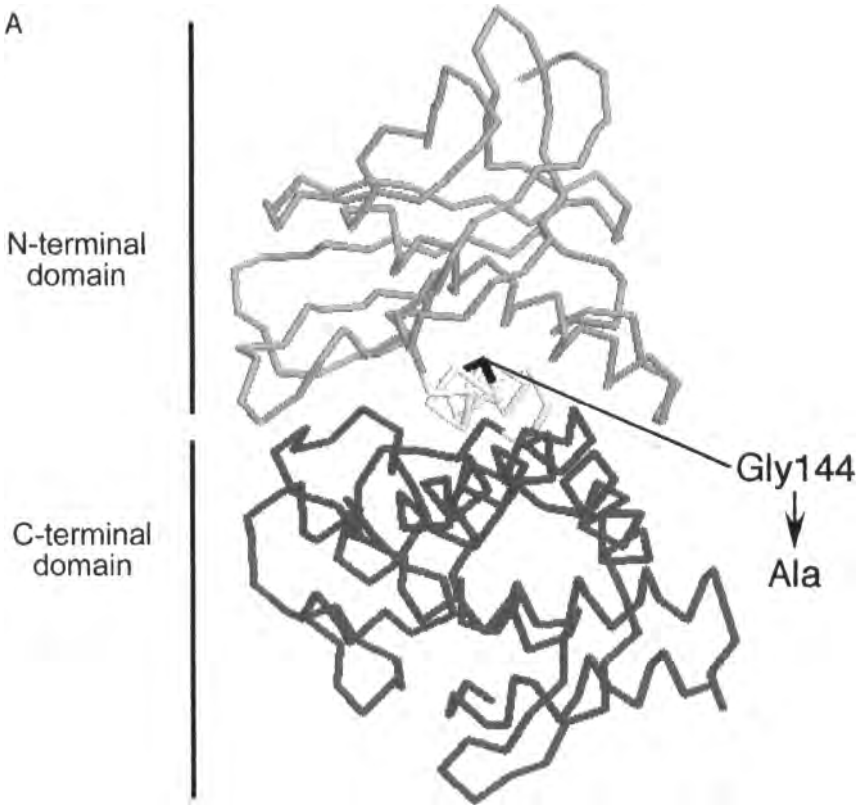
Figure 3-3. Hydrogen bonds between the tyrosyl-tRNA synthetase and tyrosyl adenylate.

Table 3-1. Relative binding energies of groups in tyrosyl-tRNA synthetase infered from comparison between mutant and wilde-type enzymes at 298 K.

Compared residues and their numbering		Substrate	$\Delta\Delta G_T^{\neq}$ (kcal mol^{-1})
Phe 34	Tyr 34	Tyr	0.52
Gly 35	Cys 35	ATP	1.14
Ala 51	Cys 51	ATP	0.47
Gly 48	Asn 48	ATP	0.77
Gly 48	His 48*	ATP	0.96
Ser 35	Cys 35*	ATP	1.18
Phe 169	Tyr 169*	Tyr	3.72
Gly 195	Gln 195*	Tyr	4.49
Gly 35	Ser 35	ATP	– 0.04
Ala 51	Thr 51*	ATP	– 0.44

* Residues found in the wild-type protein

charged donor or acceptor weakens binding by a further 3 or more kcal mol^{-1}. These values are much lower than the absolute strength of hydrogen bonds *in vacuo* and are the consequence of hydrogen bonding in aqueous solution being an exchange process.



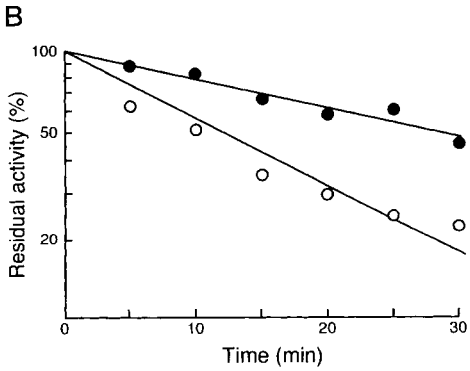


Figure 3-4. (A) Structure of thermolysin from *Bacillus thermoproteolyticus* used to determine candidate residues for site-directed mutagenesis of the neutral protease from *B. stearothermophilus*. The structure was visualized with Ras-Mol software and slightly modified. Data were from MMDB ID: 3638, PDB ID: 1LNF, submitted by Holland, and Matthews. The two domains are indicated with the bars on the left, and the Gly 144 residue is highlighted in black. (B) Thermostability of the wild-type neutral protease (open circles), and the G144A variant (closed circles).

3.6.2
Enhancing the Thermostability of Proteases

An increase in thermostability of a neutral protease from *Bacillus stearothermophilus* (NprT) was achieved from a rational approach by comparing its sequence with the thermostable thermolysin from *B. thermoproteolyticus*[33]. The enzymes were 85% identical, while the thermostability of NprT at 75 C was significantly lower than that

of thermolysin. Taking into account the statistical data of various amino acid substitutions that increase thermostability, and the three-dimensional structure of thermolysin, a single mutation G144A was chosen as a candidate to increase the thermostability of NprT. The glycine residue was supposed to be located in an α-helix that connected the *N*- and *C*-terminal domains of the enzyme (Fig. 3-4 A). The mutation was expected to stabilize the α-helix, and increase internal hydrophobicity of the enzyme. Furthermore, the G144A mutation introduces only a small methyl group, minimizing any structural or functional interruption that may be caused by introduction of a new side chain. Indeed, this single mutation led to a significant increase in the thermostability of NprT (Fig. 3-4 B). This is a good example of the fact that an increase in internal hydrophobicity of an enzyme and stabilization of a secondary structure α-helix leads to an increase in the thermostability of a protein.

3.6.3
Contribution of Ion Pairs to the Thermostability of Proteins from Hyperthermophiles

Proteins found in hyperthermophiles display an astonishing resistance to thermal denaturation. Some are stable for hours or even days at temperatures near to the boiling point. This has attracted much attention as these proteins are promising candidates themselves as stable biocatalysts, and also provide valuable hints to the understanding of the mechanisms of protein thermostability.

The present authors have pursued attempts to elucidate the three-dimensional structures of various proteins from the hyperthermophilic archaeon *Thermococcus kodakaraensis* KOD1. These include DNA polymerase[22, 23], homing endonuclease II[34], O^6-methylguanine-DNA methyltransferase (MGMT)[20, 21], aspartyl-tRNA synthetase[35], and ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco)[24, 25]. MGMT repairs alkylated DNA by suicidal alkyl transfer from guanine O^6 to its own cysteine residue. We determined the three-dimensional structure of MGMT from *T. kodakaraensis* KOD1 (*Tk*-MGMT) at 1.8 Å resolution[21]. This structure was compared with its counterpart from *Escherichia coli* (AdaC, *C*-terminal fragment of Ada protein). It has been reported that helical conformation is stabilized by (i + 4) or (i + 3) glutamate-lysine intra-helix ion-pairs in a short model peptide. We observed seven intra-helix ion pairs in *Tk*-MGMT, while none were detected in AdaC. It is presumed that these intra-helix ion-pairs contribute to reinforcement of the stability of the α-helices. Furthermore, four extra inter-helix ion-pairs not found in AdaC were observed in the interior of *Tk*-MGMT, stabilizing the internal packing of the tertiary structure. The structure of *Tk*-MGMT strongly indicates that intra-helix and inter-helix ion-pairs provide a major contribution to the thermostability of the protein.

As the importance of ion-pairs toward protein thermostability has been stressed in many cases, addition or removal of an ion-pair should have significant effects. A clear example is provided by mutagenesis studies of glutamate dehydrogenase from *T. kodakaraensis* KOD1 (*Tk*-GDH)[36]. The GDH from *Pyrococcus furiosus* (*Pf*-GDH) and *Tk*-GDH are 83% identical in terms of primary structure. However, while *Pf*-GDH displays a half-life of 12 h at 100 C, that of *Tk*-GDH is 4 h. The three-

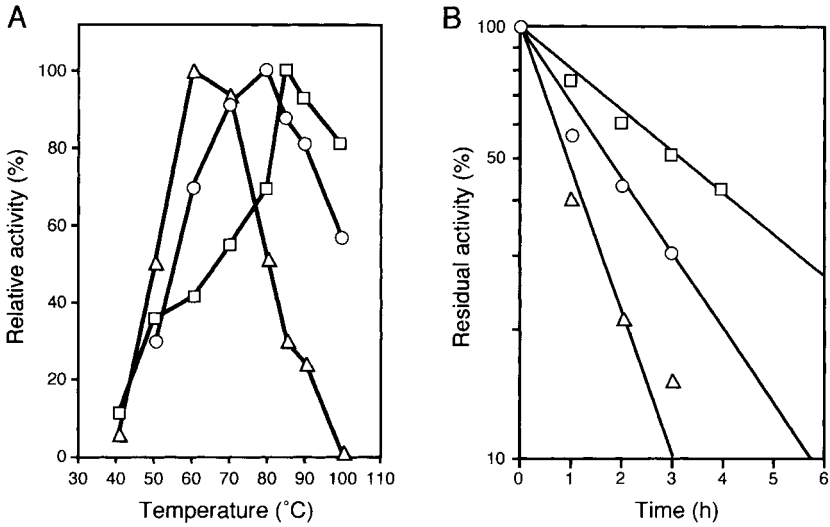


Figure 3-5. Temperature profile (A) and thermostability (B) of glutamate dehydrogenase from *T. kodakaraensis* KOD1 and its mutants. Data of the wild-type enzyme (circles), the T138E mutant protein (squares), and the E158Q mutant protein (triangles) are shown.

dimensional structure of *Pf*-GDH has been determined, and exists in a stable hexameric form. A structural model of *Tk*-GDH was constructed based on the structure of *Pf*-GDH. A difference was observed between the two structures at the monomer-monomer interface. In *Pf*-GDH, there is a large ion-pair network comprised of six residues, Arg 35, Asp 132, Glu 138, Arg 164, Arg 165, and Lys 166. Glu 138 is located at the center of the network, interacting with Arg 165 and Lys 166. In the case of *Tk*-GDH, Glu138 was replaced by a threonine residue. When a T138E mutation was introduced into *Tk*-GDH, an increase in both thermostability (2 to 3 h at 100 C) and optimal temperature (80 to 85 C) was observed, confirming the importance of ion-pair networks (Fig. 3–5). At one of the two-fold axes of the proteins, Glu 158 is at the center of another ion-pair network, interacting with Arg 124 and Arg 128. An E158Q mutation would interrupt this network, and is presumed to destabilize the protein. As expected, the E158Q mutant protein of *Tk*-GDH displayed a lower optimal temperature for activity (80 to 60 C), and decreased thermostability (2 h to 50 min at 100 C, Fig. 3-5).

3.6.4
Thermostability Engineering Based on the Consensus Concept

The examples mentioned above have shown that in many cases, sequence comparisons between two homologous enzymes with different thermostabilities provide valuable clues as to the how to increase protein thermostability rationally. An interesting observation has recently been made that even a set of amino acid sequences of homologous, mesophilic enzymes provides sufficient information to

allow the rapid design of a thermostabilized variant of the family of enzymes[37]. Using myo-inositol hexakisphosphate phosphohydrolase (phytase) as the target enzyme, a sequence alignment of 13 homologous fungal phytases was used to calculate a consensus amino acid sequence. An amino acid that has already been proven to fit into the structure of at least one of the homologous enzymes used in the alignment is chosen as a consensus residue. A synthetic gene, corresponding to the consensus phytase sequence was expressed and the recombinant protein, consensus phytase-1, was characterized. Differential scanning calorimetry revealed that consensus phytase-1 displayed an unfolding temperature (T_m) of 78.0 C, which was 15–22 C higher than the T_m values of all parent phytases used in its design. Furthermore, by including six more sequences in the alignment, a refined consensus sequence was calculated (consensus phytase-10). Consensus phytase-10 displayed even higher thermostability, with a T_m value of 85.4 C. Further optimization through site-directed mutagenesis eventually led to consensus proteins with unfolding temperatures of up to 90.4 C. When the effects of individual substitutions were evaluated, all single mutations affected the thermostability by less than 3 C. This suggests that the increases in stability observed in the consensus phytases were due to the combination of multiple amino acid exchanges distributed over the entire sequence of the protein. Remarkably, in spite of the increase in thermostability, catalytic activity at 37 C was not compromised. Although further examination with other proteins will be necessary, the consensus concept may provide a powerful alternative as a means to enhancing the thermostability of proteins when the information available is limited.

3.6.5
Changing the Optimal pH of an Enzyme

Various thermostable alcohol dehydrogenases have been studied for use in the industrial production of alcohol. Based on the three-dimensional structure of horse liver alcohol dehydrogenase and a multiple sequence alignment of alcohol dehydrogenases from various sources, the optimal pH of a thermostable alcohol dehydrogenase (ADH-T) from *Bacillus stearothermophilus* NCA 1503 was rationally shifted[38]. The amino acid residues responsible for the catalytic activity of horse liver ADH had been clarified on the basis of its three-dimensional structure. As the catalytic amino acid residues were fairly conserved in ADH-T and other ADHs, ADH-T was presumed to harbor the same proton release system as horse liver ADH, and confirmed by site-directed mutagenesis. In ADH-T, catalysis was shown to be performed by a proton release system involving a zinc-bound water molecule, a hydroxyl group of Thr 40, and an imidazole ring of His 43 (Fig. 3-6)[39]. Cys 38, which interacts with the zinc ion, along with Thr 40, and His 43 were the targets for site-directed mutagenesis, and C38S, T40A, T40S, and H43A mutants were produced. The C38S, T40A, and H43A mutations completely abolished the activity of ADH-T, while the T40S mutant displayed a slightly lower activity than the wild-type enzyme. As the pK_a value of His 43 was presumed to play an important role in proton release, an H43R mutation was incorporated in order to alter the optimal pH

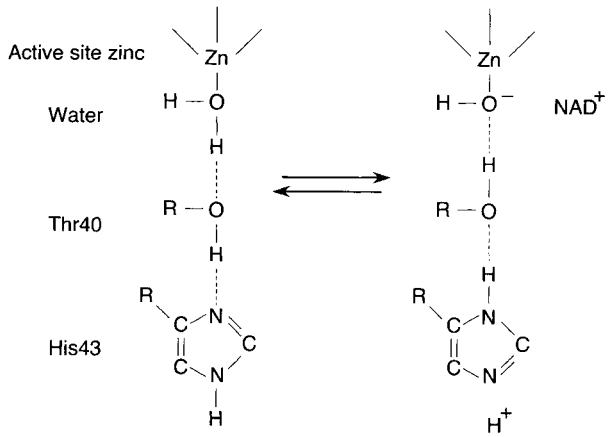


Figure 3-6. Mechanism for the proton release system of ADH-T. The system is composed of a zinc-bound water molecule, and the side chains of residues Thr 40 and His 43. Proton release is induced by NAD^+ binding.

of the enzyme. As expected, the optimum pH of the mutant enzyme H43R was shifted from 7.8 (wild-type enzyme) to 9.0. Furthermore, at the optimum pH, the H43R enzyme exhibited a higher level of activity than the wild-type ADH-T.

3.6.6
Changing the Cofactor Specificity of an Enzyme

Nicotinamide adenine dinucleotide (NAD^+) and nicotinamide adenine dinucleotide phosphate ($NADP^+$) are ubiquitous redox cofactors involved in a huge variety of enzyme reactions. The two are similar in structure, with $NADP^+$ harboring a single additional phosphate group esterified to the 2'-hydroxyl group of the AMP moiety. However, enzymes found in nature usually display a clear preference for one of the two cofactors, providing an interesting example of molecular recognition by enzymes.

Many studies have addressed this subject, and it is now possible to engineer and switch rationally the cofactor specificities of particular enzymes from $NADP^+$ to NAD^+ and *vice versa*. Pioneering work has been carried out with glutathione reductase, a member of the highly homologous flavoprotein disulfide oxidoreductase family[40]. Most members of this enzyme family utilize $NADP^+$ as a cofactor with one exception, the NAD^+-dependent dihydrolipoamide dehydrogenases. Using the three-dimensional structure of glutathione reductase from human erythrocytes (H-GR), and sequence alignment of various enzymes in the flavoprotein disulfide oxidoreductase family (Fig. 3-7), the cofactor specificity of glutathione reductase from *E. coli* (E-GR, *gor* gene product) was switched from $NADP^+$ to NAD^+. From the structure of H-GR, a βαβ "fingerprint" motif was found in the NADP-binding domain of the enzyme. Two arginine residues in H-GR, Arg 218 and Arg 224, bind to the 2'-phosphate group of the $NADP^+$ molecule. These residues are conserved in virtually all $NADP^+$-dependent enzymes in the flavoprotein disulfide oxidoreductase family, but not in the NAD^+-dependent dihydrolipoamide dehydrogenases. Substitution of each corresponding arginine residue in E-GR (R198M, R204L) or both,

```
E-GR  174  GAGYIAVELAGVINGLG---AKTHLFVRKHAPLRSFD  (NADP+)
H-GR  194  GAGYIAVEMAGILSALG---SKTSLMIRHDKVLRSFD  (NADP+)
P-GR  172  GGGYIAVEFASIFNGLG---AETTLLYRRDLFLRGFD  (NADP+)
S-MR  257  GSGYIAAELGQMFHNLG---TEVTLMQRSERLFKTYD  (NADP+)
P-MR  277  GSSVVALELAQAFARLG---SKVTVLARNTLFFRE-D  (NADP+)
T-TR  195  GGGFISVEFAGIFNAYKPNGGKVTLCYRNNPILRGFD  (NADP+)
           * *  *   *      *        **       *
E-DD  180  GGGILGLEMGTVYHALG---SQIDVVEMFDQVIPAAD  (NAD+)
B-DD  183  GGGYIGIELGTAYANFG---TKVTILEGAGEILSGFE  (NAD+)
Y-DD  209  GGGIIGLEMGSVYSRLG---SKVTVVEFQPQIGASMD  (NAD+)
H-DD  220  GAGVIGVELGSVWQRLG---ADVTAVEFLGHVGGVGI  (NAD+)
```

Figure 3-7. Sequence alignment of various enzymes in the flavoprotein disulfide oxidoreductase family. The sequences of the $NADP^+$-dependent enzymes are the glutathione reductase from *E. coli* (E-GR), human (H-GR), *Pseudomonas aeruginosa* (P-GR), mercuric reductase from *Staphylococcus aureus* (S-MR), *P. aeruginosa* Tn 501 (P-GR), and trypanothione reductase from *Trypanosoma congolense* (T-TR). The NAD^+-dependent enzymes are dihydrolipoamide dehydrogenase from *E. coli* (E-DD), *B. stearothermophilus* (B-DD), yeast (Y-DD), and human (H-DD). Residue positions marked with an asterisk correspond to those that were targets of site-directed mutagenesis in the text.

resulted in a modest fall in the k_{cat} value of the $NADP^+$-dependent activity, but caused a large increase in K_M toward $NADP^+$ (~25-fold). Drastic effects were not observed for NAD^+-dependent E-GR activity.

Further mutations were introduced, focusing on the G-X-G-X-X-G motif, found in various NAD^+-dependent dehydrogenases, including dihydrolipoamide dehydrogenase. In $NADP^+$-dependent enzymes, including H-GR and E-GR, the third Gly residue is usually replaced by an Ala residue (Ala 179 in E-GR). Another Ala residue is also conserved four residues further toward the *C*-terminus in $NADP^+$-dependent enzymes (Ala 183 in E-GR), but substituted by a Gly residue in dihydrolipoamide dehydrogenases. The A179G mutation in E-GR led to a dramatic decrease in the K_M toward NAD^+ (~40-fold), with little change in the k_{cat} value. The A183G mutation had little effect towards NAD^+-dependent activity.

Another set of mutations were introduced centered on the Val 197 residue of E-GR. In the NAD^+-dependent dihydrolipoamide dehydrogenases, this residue is replaced by a Glu residue, whose negative charge interacts with the 2'-hydroxyl group of NAD^+ via a hydrogen bond. In order to generate such interaction in E-GR, a V197E mutation, along with K199F and H200D mutations to remove residual positive charges that may interact with the 2'-phosphate group of $NADP^+$, were introduced. The mutant protein with seven mutations, A179G/A183G/V197E/R198M/K199F/H200D/R204P displayed a ~250-fold decrease in k_{cat}/K_M value for $NADP^+$-dependent activity, while that for NAD^+-dependent activity increased by a factor of ~70. The ratio of these two contrasting shifts is 18 000, indicating that the cofactor specificity of the enzyme was rationally switched.

As all mutation sites chosen in this study are limited to the βαβ "fingerprint" motif, the strategy applied is applicable to other NAD^+- and $NADP^+$-dependent dehydrogenases. Indeed, a systematic replacement of amino acid residues in the βαβ "fingerprint" motif in the NAD^+-dependent dihydrolipoamide dehydrogenase from *E. coli* converted its cofactor specificity from NAD^+ to $NADP^+$ [41]. A similar strategy

has been successfully applied on inverting the cofactor specificity of NAD^+-dependent malate dehydrogenase from *Thermus flavus*, using the crystal structure of the NAD^+-dependent porcine enzyme and alignment with the $NADP^+$-dependent enzyme from chloroplasts[42]. The engineered mutant protein displayed a 1000-fold improvement toward $NADP^+$ and a 600-fold decrease in efficiency with NAD^+. Other key examples have been shown with decarboxylating dehydrogenases, isocitrate dehydrogenase (IDH)[43, 44] and isopropylmalate dehydrogenase (IMDH)[45]. Although these enzymes do not bind the nucleotide cofactors in the βαβ binding motif mentioned above, conversion of an $NADP^+$-dependent IDH into an NAD^+-dependent enzyme (850-fold preference) has been achieved[43]. Engineering the secondary structure of NAD^+-dependent IMDH from *Thermus thermophilus* led to a 1000-fold preference for $NADP^+$[45].

3.6.7
Changing the Substrate Specificity of an Enzyme

Recently, there have been an increasing number of reports where rational mutageneses of enzymes led to a dramatic change in their substrate specificity. One example is the study on cucumber linoleate 13-lipoxygenase[46]. Lipoxygenases constitute a family of non-heme, iron-containing dioxygenases catalyzing the regio- and stereoselective dioxygenation of polyenoic fatty acids to form hydroperoxy derivatives. Enzymes from plants are classified into 9- and 13-lipoxygenases according to their positional specificity toward linoleic acid oxygenation. Multiple sequence alignments and structural modeling of enzyme-substrate interaction suggested that a single residue, His 608, played a key role in the regiospecificity of the 13-lipoxygenase. An H608V mutation was introduced, and resulted in an enzyme variant with specific 9-lipoxygenase activity. This was elegantly explained by the fact that an H608V mutation enables a positively charged guanidino group of Arg 758, masked by the bulky His 608 residue in the wild-type enzyme, to interact with the carboxyl group of the substrate linoleic acid. This interaction forces a reversal of the substrate in the active site. This explanation was strongly supported by the observations that an R758L/H608V double mutant protein exhibited a lower reaction rate and random positional specificity. Furthermore, the drastic alteration of positional specificity was not observed when substrates lacking a free carboxyl group were examined.

Another example deals with the mammalian 3α-hydroxysteroid dehydrogenase[47]. Mammalian hydroxysteroid dehydrogenases convert potent steroid hormones into their cognate inactive metabolites and belong to the aldo-keto reductase superfamily. Although 3α- and 20α-hydroxysteroid dehydrogenases display 67% amino acid sequence identity with one another, they differ in their regiospecificity and stereospecificity. 3α-Hydroxysteroid dehydrogenase converts 5-dihydrotestosterone into 3-androstanediol, while 20α-hydroxysteroid dehydrogenase converts progesterone into 20-hydroxyprogesterone, the two enzymes catalyzing the formation of secondary alcohols on opposite ends of steroid hormone substrates. The crystal structure of 3α-hydroxysteroid dehydrogenase complexed with testosterone indicated that 10 residues located on 5 loop structures were involved in the enzyme-substrate

interaction. Multiple sequence alignment of various hydroxysteroid dehydrogenases displayed that 6 of these 10 residues were substituted in the 20α-enzyme. Single and multiple replacements of the 3α-enzyme residues to the 20α-enzyme residues did not lead to an alteration in regiospecificity. However, when individual loops were exchanged, a drastic change in regiospecificity was observed. An exchange of loop A led to a protein variant with both 3α- and 17β-hydroxysteroid dehydrogenase activity. A double exchange of loops A and C resulted in 3α- and 20α-activity. Finally, a triple exchange of loops A, B and C completely converted the specificity of the enzyme into a stereospecific 20α-hydroxysteroid dehydrogenase with a resultant shift in k_{cat}/K_M for the desired reaction of 2×10^{11}.

3.6.8
Changing the Product Specificity of an Enzyme

A rational approach can also be used to change the product specificity of an enzyme. Prenyl diphosphate synthases catalyze the condensations of isopentenyl diphosphate with allylic diphosphate to give linear hydrocarbons of various lengths and different stereochemistries. Heptaprenyl diphosphate synthase from *B. stearothermophilus* is a member of the medium-chain prenyl diphosphate synthases. The enzyme catalyzes the consecutive condensation of isopentenyl diphosphate with allylic diphosphate to produce (all-*E*)-C35 prenyl diphosphate as the ultimate product. The product specificity of short-chain prenyl diphosphate synthases has been shown to be regulated by a structure around the first aspartate-rich motif (FARM). Component II' of heptaprenyl diphosphate synthase also harbors a FARM, suggesting that this structure in component II' may also regulate elongation in this enzyme. Via site-directed mutagenesis, a relatively bulky isoleucine residue eight positions before the FARM, was substituted by a small glycine residue (I76G variant). As anticipated, the I76G variant catalyzed condensations of isopentenyl diphosphate beyond the native chain length of C35. Furthermore, two small residues Ala 79 and Ser 80 were individually replaced with the bulky tyrosine and phenylalanine, respectively (A79Y and S80F variants). In contrast to the I76G mutation, these variants mainly yielded a C20 product. The study demonstrates that in the wild-type enzyme, the elongation reaction is precisely blocked at the length of C35 by the bulky Ile 76 residue, and that the degree of elongation can be controlled by removal or introduction of a bulky residue in the enzyme pocket[48] (Fig. 3-8).

A similar approach can be utilized with the geranylgeranyl diphosphate synthase from *Sulfolobus acidocaldarius*. The wild-type enzyme yields (all-E)-C20 prenyl diphosphate as a final product. The three-dimensional model of the enzyme suggested that the removal of two bulky residues Phe 77 and His 114 would allow additional prenyl-chain elongation. F77G, F77G/H114A, F77G/H114G, H114A, and H114G variants gave C30, C(45), C50, C30 and C40 as the major maximum length products, respectively[49].

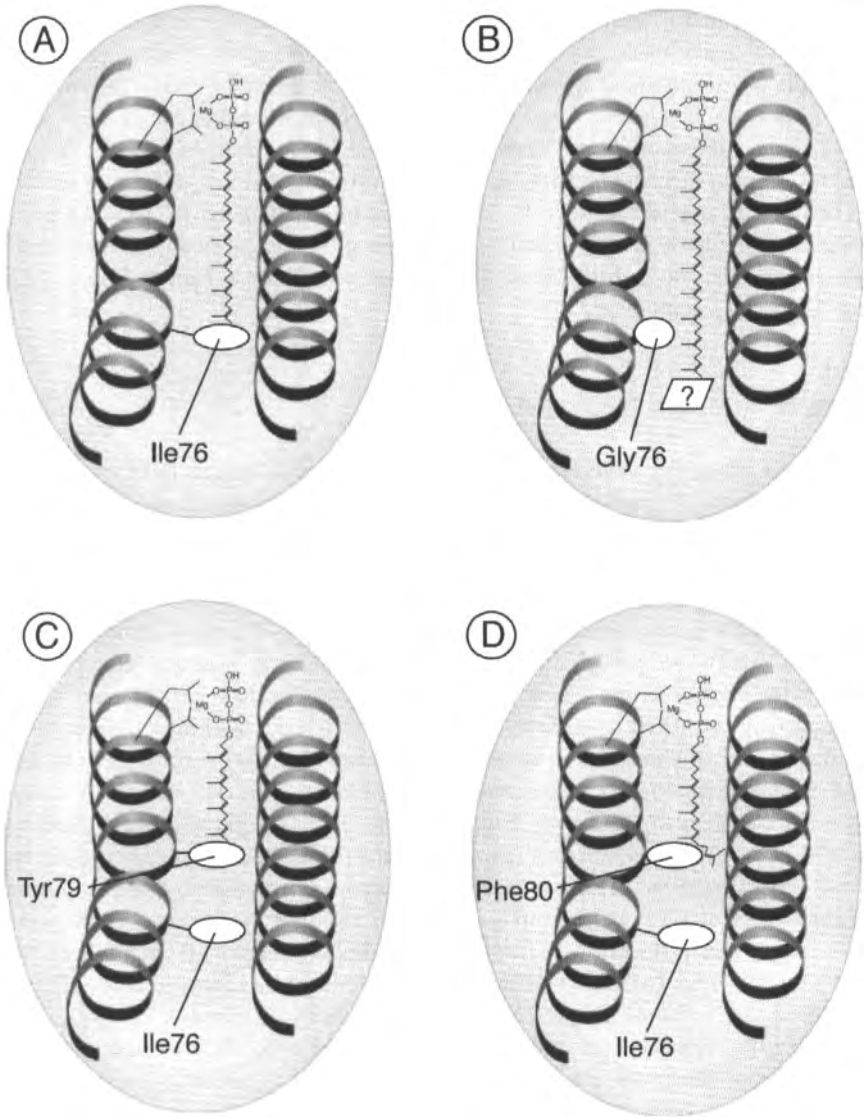


Figure 3-8. Proposed mechanism of the chain-length determination of the wild-type and variant heptaprenyl diphosphate synthases based on the pocket mechanism. A, Wild-type enzyme; B, I76G variant; C, A79Y variant; D, S80F variant.

3.6.9
Combining Site-directed Mutagenesis with Chemical Modification

Combining site-directed mutagenesis strategies with chemical modification is a popular tool in both enzyme engineering and mechanistic studies. This has often been applied to the subtilisin from *Bacillus lentus* (SBL), or savinase. Subtilisins are

one of the most well-characterized and well-engineered proteins; mutational effects of more than half of the 275 amino acid residues have been reported[50]. A high-resolution, three-dimensional structure of SBL is also available. Furthermore, wild-type SBL does not harbor any cysteine residues. Therefore, if a single cysteine residue were to be introduced by site-directed mutagenesis, treatment with methanethiosulfonate reagents would lead to specifically localized modification of the enzyme. This approach, the chemically modified mutant approach (CMM), has been utilized in altering the stability, specificity, kinetic properties, and pH profiles of SBL. The following example displays how the CMM approach can expand the specificity of the S1 pocket of SBL[51].

Wild-type SBL is known to prefer bulky, hydrophobic P1 residues in its S1 pocket. The Phe P1 residue of the standard suc-AAPF-pNA (succinyl-alanyl-alanyl-prolyl-phenylalanyl-*p*-nitroanilide) substrate was shown to be preferred by a factor of 9500-fold over the small P1 residue of suc-AAPA-pNA, by a factor of 24-fold compared with the positively charged P1 residue of suc-AAPR-pNA and by a factor of 522-fold compared with the negatively charged P1 residue of suc-AAPE-pNAA. The Ser 166 residue, located at the bottom of the S1 pocket and whose side chain points inward toward the pocket, was chosen for substitution by cysteine and subsequent chemical modification. In order to increase specificity toward small uncharged P1 residues such as Ala, bulky moieties, for example benzyl, decyl, cyclohexyl, and steroidyl groups, were incorporated at S166C so as to reduce the volume of the S1 pocket and induce a better fit for small P1 groups. Likewise, negatively charged groups such as an ethylsulfonato moiety, a dicarboxylic aromatic group, and aliphatic mono-, di-, and tri-carboxyl groups were incorporated for higher specificity for positively charged P1 residues such as Arg. A positively charged ethylamino group was introduced to improve the acceptance of the negatively charged P1 residue Glu.

In the case of a cyclohexyl group, the modified enzyme showed a 2-fold improvement in k_{cat}/K_M with the suc-AAPA-pNA substrate and a 51-fold improvement in suc-AAPA-pNA/suc-AAPF-pNA selectivity relative to WT-SBL. The enzymes modified with mono-, di-, and tricarboxyl groups displayed improved k_{cat}/K_M values toward suc-AAPR-pNA. Furthermore, these values increased in parallel with the number of carboxyl groups introduced, and led to a 9-fold improvement in k_{cat}/K_M for the suc-AAPR-pNA substrate and a 61-fold improvement in suc-AAPR-pNA/suc-AAPF-pNA selectivity compared with the wild-type SBL. Conversely, the introduction of the positively charged ethylamino group led to a 19-fold improvement in k_{cat}/K_M for the suc-AAPE-pNA substrate and a 54-fold improvement in suc-AAPE-pNA/suc-AAPF-pNA selectivity relative to the wild-type SBL.

3.6.10
Changing the Catalytic Activity of a Protein

With the abundant number (> 16 000) of three-dimensional structures in the Brookhaven Protein Data Bank, a challenging but promising task in protein engineering is the synthesis of novel biocatalysts by assembling individual functional modules (substrate binding sites, catalytic centers etc.), or by introducing a

designed functional environment into a known protein template structure. The following is an example of the latter strategy, taking advantage of the diverse functions of a protein superfamily sharing a common fold[52].

The 2-enoyl-CoA hydratase/isomerase enzyme superfamily is comprised of enzymes with various specificities and functions, including 4-chlorobenzoyl-CoA dehalogenase, 2-enoyl-CoA hydratase, carnitine racemase, dihydroxynaphthoate synthase, 2-ketocyclohexanecarboxyl-CoA hydrolase, Δ3,Δ2-enoyl-CoA isomerase, and even the proteolytic component of Clp protease. Structural comparison of these proteins indicated the possibility that a majority of the individual active sites were derivatives of a single active site structure. This environment provides a CoA binding site, an expandable acyl-binding pocket, an oxyanion hole for binding or polarizing the thioester carbonyl group, and numerous sites for strategic positioning of catalytic residues. In the study, the active site of one member of the 2-enoyl-CoA hydratase/isomerase family, 4-chlorobenzoyl-CoA dehalogenase, was altered by site-directed mutagenesis to include the two glutamate residues functioning in acid/base catalysis in a second family member, 2-enoyl-CoA hydratase. As a result, the *syn* hydration of 2-enoyl-CoA, absent in the wild-type 4-chlorobenzoyl-CoA dehalogenase, was observed in the engineered protein with k_{cat} and K_M values of 0.06 s^{-1} and 50 M, respectively. Although the efficiency of the engineered protein is far from the native 2-enoyl-CoA hydratase, the study clearly demonstrates the possibility of exchanging catalytic functions of two enzymes within a structural enzyme family. It also sends an encouraging message that if an appropriate template is available, it is possible to obtain a desired enzyme activity by rationally designing a catalytic environment on the "template landscape".

Other studies have explored or resulted in even more drastic alterations in enzymatic characteristics. Tyrosine phenyl-lyase (TPL) and aspartate aminotransferase (AspAT) both belong to the α-family of vitamin B_6-dependent enzymes. While TPL catalyzes the β-elimination reaction of L-tyrosine, AspAT catalyzes the reversible transfer of an amino group between dicarboxylic amino acids and their corresponding 2-oxo acids. The double mutation R100T/V283R, leading to an AspAT-like sequence, was introduced into TPL. The protein obtained displayed a 10^4-fold increase in β-elimination activity towards dicarboxylic amino acids than the wild-type TPL. The activity towards L-aspartate was twice as high as that towards the native substrate L-tyrosine. The created enzyme can be considered a dicarboxylic amino acid β-lyase, an enzyme that is not found in nature[53]. A further study attempted to design a protein with enzymatic activity, starting from a structurally homologous non-catalytic protein scaffold[54]. The nuclear transport factor 2 (NTF2) and scytalone dehydratase both share a common α/β barrel structure. Four key catalytic residues, along with a C-terminal α-helix found in scytalone dehydratase, but not in NTF2, were introduced into the NTF2 protein. A mutant protein exhibited scytalone dehydratase activity with minimal k_{cat} and K_M values of 0125 min^{-1} and 800 μM, respectively. The study is one of the few examples of converting a non-catalytic protein scaffold into an enzyme.

3.7 Conclusions

The examples above represent some of the most successful studies in protein engineering. They show that it is possible to enhance protein thermostability rationally, alter cofactor or substrate specificity, regiospecificity, and even change catalytic activity. Furthermore, the creation of enzymatic activity from a non-catalytic protein backbone, and the creation of a biocatalyst with an unprecedented catalytic activity not found in nature, have also been achieved. However, the examples published in the literature are probably only a tiny fraction of the many studies that have been, or are still, in progress awaiting positive results.

We are still at a premature stage in designating precise rules to engineer a variant protein with each and every desired property. It is still not easy to predict the outcome of even a single amino acid residue substitution. However in some cases, depending on the information available and the property desired, some basic guidelines are available. Whatever the position, the three-dimensional structure of the protein, or of a homologous protein is highly desired. Without any structural information, strategies will be limited, and the sense of rationality of the experiments will be low.

When enhancement of protein (thermo)stability is desired, there are a number of strategies available, taking into account four major interactions within a protein; covalent bonds via disulfide bridges, ionic interactions, hydrogen bonds, and hydrophobic interaction (Fig. 3-9). Introducing a covalent disulfide bond in a region distant from the catalytic center of T4 lysozyme was reported to enhance dramatically the thermostability of the protein[55, 56]. With human lysozyme, introduction of Asp residues to generate a Ca^{2+} binding pocket rationally, and consequently ionic interactions, led to a calcium binding variant protein with an increase in thermostability[57]. Although performed by a random approach, the effects of hydrogen bonds on protein thermostability has also been displayed with T4 lysozyme[58]. A single T157I mutation, interrupting a hydrogen bond in the wild-type enzyme, led to a temperature-sensitive mutant protein. The importance of hydrophobic interactions has been mentioned above. Addition of any of these four types of interactions may be considered in order to enhance the thermostability of a protein. Another alternative may be to introduce proline residues at β-turn structures (the proline rule). This has been clearly demonstrated with oligo-1,6-glucosidases from various *Bacillus* species[58–61].

When the aim is to alter the substrate or cofactor specificity of an enzyme, one should look for a homologous structure of an enzyme bound with the target molecule or a structurally similar compound (template structure). This will provide much more information than the structure of a homologous protein alone, even when the latter has been determined at a higher resolution. If the (modeled) structure of the target enzyme is also available, superimposing the structures of the two proteins will make the examination of the supposed interaction of the target enzyme and the binding molecule possible. Side chains that sterically or electrostatically interfere with binding may be identified, and subsequent mutations can be

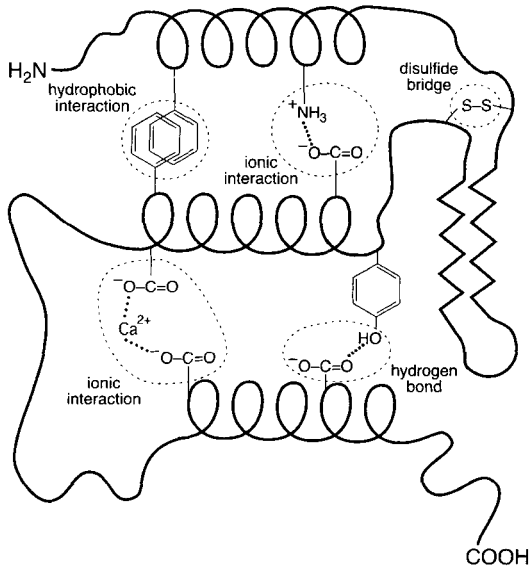


Figure 3-9. Various interactions in a protein molecule. Increasing these interactions may enhance the thermostability of the protein, as described in some examples in the text.

designed for their removal. On the other hand, residue substitutions that may possibly enhance affinity or increase specificity can also be designed. Even when a (modeled) structure of the target protein is not available, an accurate sequence alignment may also be sufficient, as long as the three-dimensional template structure is available. In some very well-studied cases, such as the βαβ binding motifs for NAD(P) cofactor binding (mentioned above), primary sequence alignment may provide enough information to engineer the binding site.

Recent studies, some mentioned here, convey new strategies and concepts for protein engineers. Combining rational design with directed evolution has also become a popular means of obtaining a protein with a desired function. The growing number of strategies will surely attract more scientists to become engaged in the field of protein engineering. This will hopefully accelerate the accumulation of information available to the engineer, ultimately enabling the *de novo* design of a biocatalyst.

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4
Enzyme Engineering by Directed Evolution

Oliver May, Christopher A. Voigt and Frances H. Arnold

4.1
Introduction

Previous chapters have outlined the huge potential of enzymes as tools for organic synthesis. However, this potential is only slowly being realized in large-scale industrial applications. The main reason for this is that enzymes are often incompatible with the specific requirements of a synthesis, especially under economic constraints. Enzyme behaviors such as substrate or product inhibition, stability, and catalytic efficiency (k_{cat}/K_m) are all finely tuned by natural evolution to support efficient reproduction of the organisms that make them. Product inhibition can be useful in a living cell, where it prevents the accumulation of undesired or even toxic products. But it is highly undesirable in a synthesis requiring high substrate concentrations and complete conversion into products. Similarly, an enzyme may naturally be highly substrate specific so as to prevent undesired side reactions with other chemically similar metabolites. But such an enzyme can only be used to synthesize a very limited range of products. Other properties that are highly desirable for chemical applications, such as long-term stability and activity in organic solvents, are simply not required in nature and are therefore not found in natural enzymes. While it is possible to devise effective bioprocess engineering solutions to some of these problems, it will often be necessary or more effective to engineer the catalyst itself.

The previous chapter reviews methods for structure-guided enzyme engineering. A prerequisite for this approach is knowledge of the enzyme structure and detailed insight into how this structure determines function. Then we must be able to predict how specific amino acid changes affect the desired properties. Despite rapid growth in the numbers of enzyme structures solved and the considerable progress made in computational methods, our understanding is still very limited and in most cases insufficient to obtain the desired features with an acceptable probability of success.

The strategy nature uses to adapt organisms to new demands is evolution. According to Darwinian theory, the fantastic diversity of life was created by random mutation and natural selection[1]. The power and simplicity of the evolution

algorithm has tempted scientists and engineers to try to implement this same approach for biomolecular design. In 1984, long after Eigen's pioneering work on the theory of evolution[2,3], Eigen and Gardiner suggested the following procedure that "*should allow a new type of evolutionary biomolecular engineering*"[4]:

10 PRODUCE A MUTANT SPECTRUM OF SELF-REPRODUCING TEMPLATES
20 SEPARATE AND CLONE INDIVIDUAL MUTANTS
30 AMPLIFY CLONES
40 EXPRESS CLONES
50 TEST FOR OPTIMAL PHENOTYPES
60 IDENTIFY OPTIMAL GENOTYPES
70 RETURN TO 10 WITH A SAMPLE OF OPTIMAL GENOTYPES

Scientists wishing to design useful proteins, peptides, or nucleic acids have picked up this evolutionary approach, which is now known as directed evolution, applied molecular evolution, *in vitro* evolution, or molecular breeding[5–13]. Directed evolution combines a high probability of success (the possibility of obtaining an improved catalyst within months) with no requirement for detailed knowledge of structure, function or even mechanism. The basic evolutionary engineering approach outlined in Fig. 4-1 has generated impressive results in a few short years, from enzymes that function in organic solvents[14] and at high temperature[15] to enzymes that are active towards non-natural substrates[16] or even carry out whole new reactions[17]. It is now clear that directed evolution will drive biocatalysis into a growing number of commercial settings, including many synthetic applications.

The aim of this chapter is to explain the concepts underlying directed evolution and to describe its application to engineering useful enzymes. In Sect. 4.2, we describe the principles of an evolutionary optimization algorithm. The tools and their implementation in different working strategies of directed evolution are then described in Sections 4.3 and 4.4. The intention is to highlight the main practical and conceptual differences among the various approaches and to compare their strengths and limitations. Section 4.5 discusses specific examples of directed evolution, with a focus on enzymes and properties that are of interest in organic syntheses. Many other important and highly successful applications of directed evolution, such as the design of catalytic antibodies and nucleic acids (ribozymes) or peptides and proteins of pharmaceutical interest, are covered in recent reviews[13, 18–26].

4.2
Evolution as an Optimizing Process

Without an understanding of the theory of evolution, one may be tempted to consider *in vitro* evolution an irrational, trial-and-error approach to protein design. However, the beauty of the structural architectures and sophisticated functions that nature has created attest to the power of the evolutionary design strategy. Many theoretical studies of evolution explain this process based on physical principles. The

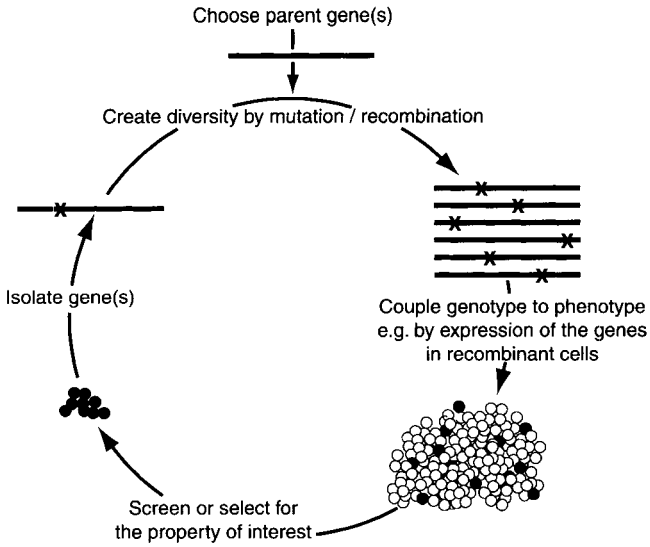

Figure 4-1. Evolutionary enzyme optimization. One or several parent genes are chosen and subjected to mutagenesis and/or recombination. The mutant gene library (genotype) is then expressed *in vivo* (or *in vitro*) where it is linked to the enzyme produced (phenotype). These enzymes are tested for the targeted property by screening or selection. DNA from the most fit clone(s) is isolated and subjected to a new cycle of mutagenesis and screening or selection. This procedure is repeated until the desired goal is reached or until no further improvements are observed.

principles that emerge are very different from those important in traditional "rational" design. Rather than trying to fully understand how mutations affect the structure and function of the enzyme (which is very difficult), the physics of evolution aims to understand the forces that make systems and problems evolvable. That is, what makes proteins so apt for evolution? Moreover, how can this be used to advantage in enzyme design?

4.2.1
The Search Space of Chemical Solutions

To describe evolution as a search process, it is necessary to define the search space. It is convenient to define sequence space as the connected network of all possible amino acid combinations (for a fixed sequence length) [27]. For a protein composed of A different amino acids and a sequence length of N residues, there are N sequences, connected by an $N(A\text{-}1)$-dimensional network. Each point in this vast space has an associated fitness, representing the combination of properties undergoing selection. Together, sequence space and a fitness description construct a fitness landscape on which an enzyme walks towards higher peaks under the influence of mutation and selection [28–30].

Exhaustively searching all possible solutions is impossible as sequence space is extraordinarily large. The mass of all amino acid combinations for 285 residues (about 10^{370} possible sequences) would be 10^{300} times the mass of the universe, thus creating sequence spaces of a size greater than the power of our imagination[31]. Nature could have explored only a small fraction of the sequence space of proteins during the age of earth. Nevertheless, excellent solutions to biological and environmental challenges have been found. Similarly, *in vitro* evolution experiments have been successful in finding improved molecules, although *a priori* success probabilities may seem to be prohibitively small. There are many successful examples where only a very small fraction of the many possible sequences have to be explored to find mutants with improved properties. The key is to develop experimental algorithms that optimize exploration subject to practical limitations.

4.2.2
The Directed Evolution Algorithm

Spontaneous mutations, recombination and selection are the tools of evolutionary design. Plant and animal breeders who influence properties of the offspring by choosing parents with the desired traits have been successfully using these tools for millennia. Following their lead, molecular biologists first employed selection strategies that acted on spontaneously generated bacterial mutants with the goal of developing new metabolic pathways[32, 33]. Solutions found by these approaches often resulted from complicated changes in regulatory genes, transport proteins, or the activation of silent genes. Whole pathways are targeted by the evolution experiment when such solutions are desired. However, if the target is a specific enzyme, it is not desirable to produce solutions that are not directly related to the enzyme.

The milestone techniques of cloning and *in vitro* recombination of genetic information[34] and other advances in molecular biology, such as the development of the polymerase chain reaction (PCR)[35], allow carefully controlled directed evolution experiments. Researchers are now able to specifically engineer the enzyme of interest and control the rate of mutagenesis and focus mutations towards specific regions within the gene. Furthermore, methods are now available to reconstruct "sexual" recombination *in vitro*[36–38] as well as in recombinant cells[39, 40]. In addition, using screening and *in vitro* selection methods, we can control the selection pressure independently of constraints in living cells, thus allowing acquisition of properties never required in nature. Technologies are available to create protein libraries of up to 10^{13} molecules and select them within a few hours or days[41].

All of these tools allow us to implement the evolutionary design algorithm *in vitro* and accelerate it to create molecules with desired properties in a fraction of the timescale of natural evolution. The success of evolutionary protein design is highly dependent on the thoughtful combination of methods for creating diversity and searching the mutant population that is generated. Optimizing an evolutionary search has been well studied in the computer science genetic algorithm literature[42, 43]. Some of these results can be applied to directed evolution, including

determining appropriate mutation and recombination rates, optimal recombination parameters, and the appropriate screening effort[44].

4.3
Creating a Library of Diverse Solutions

Given the high cost (both in terms of money and time) of analyzing a mutant library, the goal of the diversity-creating step is to produce mutant libraries that are rich in variants with improved properties. To achieve this, the few positive mutations that might occur on a gene cannot be diluted with many neutral or deleterious mutations. The level of mutant redundancy also affects the quality of the molecular diversity. Redundancy must be low because screening or selection efforts are wasted on testing identical mutants. In this section, we will first describe different approaches to creating mutant libraries, including mutation and recombination.

4.3.1
Mutagenesis

A commonly used strategy to create mutant libraries is to target the whole gene for random point mutagenesis. Nucleotide mutations are typically introduced by error-prone PCR, mutator strains, or by treatment of the isolated DNA with chemicals or UV light. The success of this approach depends critically on using an appropriate error rate. If the error rate is too low, inadequate diversity is created and screening is wasted on large numbers of redundant parent enzymes. On the other hand, if the error rate is too large, the fraction of positive mutants also becomes very low and the search for improved mutants is wasted on screening inactive clones.

A serious limitation of the random mutagenesis approach comes from the degeneracy of the genetic code and the biases of available methods, for example the preference for transitions over transversions. Together, these effects limit the amino acid substitutions that are accessible by DNA point mutations. A combination of a stepwise random mutagenesis approach with methods of focused mutagenesis and recombination can circumvent some of these limitations. The different requirements, limitations, and advantages of the most commonly employed methods are summarized in Table 4-1. In practice, a good strategy is to use a combination of methods.

4.3.1.1
Random Point Mutagenesis of Whole Genes

Before the introduction of the polymerase chain reaction (PCR)[35], point mutations were usually produced by UV radiation, by chemical treatment[45] or by using mutator strains that have an increased mutation rate compared to normal strains because of defects in their DNA-repair mechanisms[46]. Chemical mutagenesis[47], mutator strains[48, 49], and even spontaneous mutations coupled with selection in a

Table 4-1. Comparison of methods for creating genetic diversity for directed evolution.

	Requirement	Advantage	Limitation
Random point mutagenesis	None	Exhaustive	No multiple simultaneous mutations; requires multiple rounds to accumulate beneficial mutations
Focused mutagenesis	Structural information or knowledge from previous generations	Reduced library size; multiple simultaneous mutations possible	Misses possible good sites
Recombination			
– single gene	None	Recombine positive mutations; remove neutral and deleterious ones	No multiple simultaneous mutations; recreates large number of already known sequence
– family shuffling	Homologous genes	"Functional diversity"; large jumps in sequence space	Not exhaustive; limited to amino acid diversity in parental sequence space

chemostat culture[50] are still used for random point mutagenesis in directed evolution experiments. However, the dominant method is now error-prone PCR because the protocols are very straightforward, safe, fast and versatile, and allow simple adjustment of the error level. Point mutations are introduced by PCR because of erroneous incorporation of a nucleotide during the amplification of the target gene. Under normal reaction conditions, the error rate of the *Taq* DNA polymerase is about 0.001% to 0.02% per nucleotide per replication cycle[51, 52]. Although this error rate is sufficient to create libraries of large genes[53], it is too low for efficient mutagenesis of small genes. However, the fidelity of the PCR can be reduced by changing the reaction conditions (e.g., increasing the concentration of $MgCl_2$, adding $MnCl_2$ to the reaction mixture, increasing and unbalancing the concentrations of the four dNTPs, adding deoxyinosine triphosphate (dITP), increasing the concentration of *Taq* polymerase, or increasing the extension time and cycle numbers[54–58]. These methods can result in error rates as high as 2% per nucleotide position. The possibility of using of low fidelity *Taq* mutants has been described[59], but has yet to be explored experimentally.

By changing some of the PCR conditions, the error rate can be adjusted according to the gene length to produce the desired average number of amino acid substitutions. A frequently used method to estimate the level of mutation (a quality check of the diversity) is to determine the fraction of inactive clones from small samplings of the generated mutant libraries[60, 61]. However, the statistical distribution of muta-

tions (which should be narrow) cannot be estimated by this method, and the relation between the fraction of inactive clones and average number of amino acid substitutions can differ for different enzymes. Therefore, the statistical distribution of mutations and the relationship between inactive clones and the number of mutations are determined by sequencing randomly picked mutants.

Another consideration is the distribution of mutation type. Typically, there is a strong bias for transitions (A→G or T→C) over transversions (C→G or G→C), which limits the accessible amino acid substitutions. There are protocols to reduce this bias, but they do not completely eliminate it[55, 60, 62]. In addition, the structure of the genetic code limits the accessible amino acid substitutions. Depending on the specific codons, only 24–40% of the possible amino acid changes are accessible by single base substitutions[63]. Furthermore, the accessible substitutions are more likely to be conservative with similar physicochemical properties. For large genes and small error rates in whole-gene mutagenesis, it is very unlikely that two DNA mutations will occur in the same codon, dramatically reducing the possible amino acid substitutions.

Although little is known of the cost of these constraints in directed (and natural) evolution, several studies have shown that the best mutations at specific sites required multiple substitutions in a single codon[64, 65]. Methods that introduce diversity at the codon level might therefore be preferable to methods that create point mutations at the nucleotide level[63]. Methods available for codon-level mutagenesis of a few amino acid positions are unfortunately very cumbersome and expensive for mutagenizing whole genes, leaving room for future developments of improved mutagenesis methods.

4.3.1.1.1 Optimal Mutation Rates: Experimental

One rule-of-thumb has been to adjust the error rate according to the number of targeted amino acid residues and the size of the screen, such that a significant fraction of the total number of combinatorial possibilities can be sampled[66]. For an enzyme of 300 amino acid residues, there are about six thousand possible single mutants, sixteen million double mutants, and thirty billion triple mutants. It is generally within our ability to exhaustively screen a single-mutation library, whereas double-mutation libraries already exceed the throughput of most screening methods (typically $< 10^6$). Standard selection methods allow a throughput of about 10^8 mutants and can therefore sample most double mutants. *In vitro* selection methods might push this limit somewhat higher (10^{12}). The assertion that it is desired to sample most of the combinatorial possibilities is based on the assumption that beneficial mutations are rare and the probability is very small that multiple random amino acid substitutions are beneficial.

An error rate resulting in single or double mutation libraries has been found to be a good compromise between creating adequate diversity while limiting the screening effort. In practice (e.g., Table 4-3), significant improvements in enzyme activity, stability, selectivity, folding, and expression have been achieved by the stepwise accumulation of single amino acid substitutions. Even large changes in substrate

specificity[62] and inversion of enantioselectivity[65] can be achieved by this conservative approach. The obvious disadvantage of this approach is that some properties will benefit from simultaneous mutation of multiple amino acids. These solutions will not be found.

Arguments for low error rate mutagenesis were recently challenged by several researchers. Zaccolo and Gheradi produced β-lactamase libraries with error rates that they claim generated 5–16 amino acid changes per mutant enzyme per generation[67]. The libraries (~ 10^4–10^5 clones) were selected for increased resistance to the antibiotic cefotaxime over three generations. However, the best mutant differed in only three effective amino acid substitutions from wild-type and is therefore much less mutated than would be expected from three generations of >5 amino acid changes per generation. This indicates that the applied average error rate was actually much lower, and a more suitable average mutation rate would have been three amino acid substitutions. Another recent study by Georgiou and coworkers indicates that a high mutation rate was appropriate in improving the affinity of a single-chain antibody[68]. When screening for improved affinity, they found that the most improved mutants were observed in libraries created with moderate to high mutation rates (3.8–22.5 mutations/gene). In the next section, we discuss the circumstances under which a higher mutation rate is advantageous.

4.3.1.1.2 Optimal Mutation Rates: Theory

In nature, the spontaneous mutation rate is tightly controlled. During one replication round, about 3×10^{-3} mutations occur in the genome of human cells, which is about the same level as in *Escherichia coli*[69]. RNA viruses, such as HIV, have a much higher mutation rate, typically on the order of one mutation per genome per replication round[70]. Such a high mutation rate is crucial for the viruses to survive the attacks of the immune system. However, there is a maximum mutation rate, above which the requirement of inheritance for evolutionary optimization breaks down (referred to in quasi-species theory as the error threshold[71]). At the mutation rate just before this threshold, the speed of evolution is highest[3, 72]. Interestingly, it was demonstrated that the mutation rate of the replication machinery of fast evolving RNA viruses is indeed close to this error threshold[73]. For directed evolution of enzymes, the optimal mutation rate maximizes the speed of the adaptive walk and is influenced by the number of mutants that can be screened and the structure of the fitness landscape. As discussed in the previous section, the general rule has been to use a mutation rate for which the permutations can be effectively sampled during screening. However, if the fitness landscape is amenable to a high mutation rate, fewer mutants must be screened in order to achieve the benefit of a higher mutation rate. For example, it would require a smaller library than all double mutant permutations to benefit from a mutation rate of two per sequence. In this section, we explore how the optimal mutation rate is influenced by the number of mutants that can be sampled, the fitness of the parents, and the ruggedness of the fitness landscape.

The optimal mutation rate depends on fitness of the parental sequence. For a sub-

optimal sequence, a large mutation rate allows a greater sweep of sequence space. However, because the probability of finding improved mutations decreases as the fitness of the sequence increases, adaptation via a large mutagenesis rate is rapid at first, then slows. If the parent is highly optimized, the probability that a mutation is deleterious is higher. The accumulation of deleterious mutations is more rapid and these mutations quickly erode the few positive mutations that occur. By using a Markov chain analysis to study genetic algorithms, Mühlenbein found that there should be approximately one amino acid substitution per sequence for highly optimized sequences[74]. His analysis also suggested that that the optimal mutation rate should decrease as the fitness of the parent increases. In several independent studies, it was demonstrated that the solution of an evolutionary search is improved when the mutation rate was decreased over time[75–78].

A higher mutation rate dramatically increases the fraction of mutants in the library that contain stop codons, requiring a larger screening effort[79]. For instance, if the average number of DNA mutations per gene is five, over 20% of the resulting library will contain stop codons. The quality of the mutant library can also be degraded by the accumulation of deleterious mutations, an effect that is exacerbated by the landscape ruggedness[79]. For the mutation of a highly coupled residue to generate a fitness improvement, it is necessary to optimize all the other residues to which it is coupled. Ideally, the optimal mutation rate equals that of the maximum number of residues involved in a single coupled interaction, thus assuring that the sequence will not become trapped in a local optimum. However, the finite number of mutants that can be screened imposes an upper limit on the mutation rate. Therefore, the optimal mutation rate decreases as the landscape ruggedness increases. This observation is similar to the long-jump mutagenesis strategy suggested by Kauffman[80]. By making moves that are larger than the correlation length (smoother landscapes have larger correlation landscapes), more space can be explored. Quasi-species theory also predicts that smoother landscapes have higher optimal mutation rates[81].

Because real protein fitness landscapes are undoubtedly highly anisotropic, they contain many correlation lengths, and different regions of the sequence will have different optimal mutation rates[44, 82]. A highly coupled region (such as the catalytic site) has a small correlation length; thus a smaller mutation rate is allowed with a limited mutant library. Based on some simplified simulations, it was found that the probability of picking a mutant that has a highly coupled mutation decreases significantly as the sequence increases in fitness[44]. This effect intensifies as the number of interactions that are coupled to the mutated residue increases. From this observation, it follows that when the screening effort is limited, uncoupled regions of the protein should be targeted for mutation. More highly coupled residues require a larger rearrangement of amino acids than is likely given the limited mutation rate. Avoiding the regions of high coupling decreases the total number of residues undergoing mutagenesis. To utilize this observation, it is necessary to have experimental techniques to target specific positions as well as methods that can be used to predetermine the coupling of each residue. These goals are the subject of the following two sections.

4.3.1.2
Focused Mutagenesis

Focused mutagenesis strategies are used with the intention of enriching a library for desired mutants. To reduce screening efforts[83–85], the targeted region can be reduced from 300 to only a few residues (Fig. 4-2). The library of quintuple mutants has a theoretical size of only ~10^6 mutants, compared to 10^{16} if the entire gene is targeted. This reduced library can be searched exhaustively with currently available methods. Focused mutagenesis significantly reduces screening requirements for libraries of mutants with multiple amino acid substitutions and eliminates the codon bias of PCR. However, it imposes obvious limitations on the possible solutions and can fail to explore the most effective mutations.

Targeting single amino acids ("saturation mutagenesis") is straightforward because of available strategies that eliminate laborious subcloning steps. Several commercial kits are available, such as the Transformer™ (CLONTECH Laboratories, Palo Alto, CA, USA), Altered Site® II (Promega, Madison, WI, USA), and QuickChange™ (Stratagene, La Jolla, CA, USA) site-directed mutagenesis systems, which can produce targeted mutant libraries in one day. This approach has been used to target amino acid positions that random point mutagenesis identified as important for the targeted enzyme properties[64, 65]. Variants with improved proper-

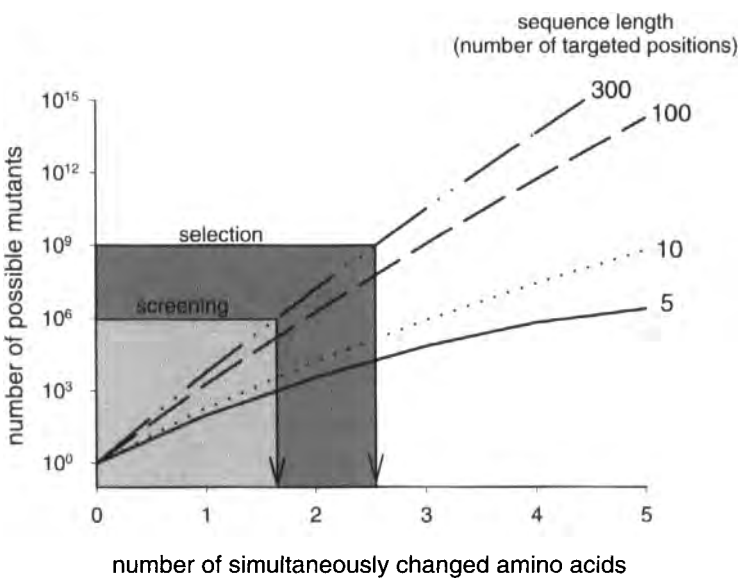

Figure 4-2. Plot showing the number of possible protein variants (Y) that can be created given the number of amino acid positions (M) changed simultaneously and the sequence length (N) ($Y=19^M[N!/(N-M)!M!]$). From a given standard screening throughput (<10^6 clones) one can calculate that it is not possible to exhaustively screen protein libraries of all possible double mutants of an average sized protein.

ties were identified in saturation mutagenesis libraries that contained mutations not accessible by random point mutagenesis.

Methods for randomization of specific regions of a gene typically employ randomized oligonucleotides or PCR mutagenesis of small stretches of the gene. The required diversity is introduced by randomized oligonucleotides that are produced by an automatic (programmable) DNA synthesizer. If only one amino acid position is targeted, the specific codon can be completely randomized (saturated) by adding an equal amount of all four bases (A, T, G ,C) during the oligonucleotide synthesis at the first two positions of the triplet and with a mixture of G and C at the third position to exclude stop codons (referred to as NN(G,C)-mutagenesis).

To target multiple amino acid positions simultaneously within a small region of the gene, oligonucleotide-cassette mutagenesis is often used. This method was among the first applied for *in vitro* evolution of DNA sequences [86] and has also been used successfully for the evolution of various enzymes [59, 87–90]. When suitable restriction sites are not already present, they are introduced by site-directed mutagenesis adjacent to the targeted region. These restriction sites are then used to substitute the wild-type region with the synthetic randomized DNA duplex (cassette) [91]. Depending on the number of targeted amino acid positions, randomization strategies are used to facilitate an exhaustive search of the library. Complete randomization of all targeted codons is preferred if few amino acid residues are targeted and can be done as described above for the randomization of single sites by using NN(C/G) during oligonucleotide synthesis. It was also reported that trinucleotide analogs such as 9-fluorenylmethoxycarbonyl (Fmoc) trinucleotide phosphoramidites can be used during DNA synthesis to achieve a codon-based mutagenesis [92].

If several distant positions are targeted simultaneously, cassette mutagenesis is technically cumbersome. Other methods allow the efficient assembly of several randomized oligonucleotides to whole genes. For example, recursive PCR [93], the ligase chain reaction [88, 94], or *in vitro* assembly of whole genes [95] can be used to construct targeted mutant libraries. Partial randomization of many positions most frequently employs spiked oligonucleotides, which are produced by DNA synthesis using a mixture of the wild-type base and equimolar amounts of all four bases [96]. The oligonucleotides can also be produced by error-prone PCR. It is possible to calculate the appropriate compositions of the nucleotide mixture in order to encode whole sets of amino acids at certain positions [97].

4.3.1.3
Calculation of Mutagenesis Hot-Spots

In focused mutagenesis experiments, the challenge is to identify the residues where mutagenesis is likely to be beneficial. Indeed, many successful directed evolution experiments show that mutations occur in regions that would be hard or impossible to predict (and difficult to explain that they do), even when a high-resolution structure and much information about the enzyme is available [14, 98–100]. One possibility is to make use of knowledge gained from early rounds of random point

mutagenesis that targeted the whole enzyme[64, 101, 102]. The content of the mutant library can be improved by only mutating sites that do not severely disrupt stability. A structurally tolerant protein allows more mutations, and therefore more potentially beneficial ones, making it more likely that there is a connected path in sequence space of single mutations that leads to regions of higher fitness. By reducing the evolutionary search to regions of sequence space that retain structure, functional space can be explored more thoroughly[82]. This concept can also be inverted: if the goal is to improve stability while retaining functionality, then eliminating the sequence space inconsistent with the function improves the search.

Several groups have proposed targeting mutagenesis to residues where natural diversity is observed. Fersht and coworkers reengineered the tumor suppressor p53 by creating a small library of mutants where the hot-spots were determined from a sequence alignment of 23 homologous proteins[103]. The mutations were made in the wild-type sequence background, and several were found that improved stability. Using a similar methodology, Lehmann et al. constructed a thermostable phytase from the consensus sequence of 13 homologous proteins[104]. The mutant phytase exhibited a 15–22 °C increase in melting temperature.

Alanine scanning has been widely used to identify the residues which are contributing to various protein properties[105, 106]. Alanine substitutions are made at various positions and the perturbation in the property of interest is measured. This has several potential applications to directed evolution. For instance, it can be used to predetermine which positions are essential to the structure (or function) of the protein and therefore should be avoided. Conversely, positions that tolerate the alanine substitutions may be good candidates for saturation mutagenesis. Unfortunately, this procedure is tedious. To surmount this difficulty, Kollman and coworkers proposed a method to determine the effects of alanine substitutions computationally[107]. Kollman's method could be used to scan the protein structure for positions to mutagenize in directed evolution.

The observation that some sequence positions are more tolerant to mutation initiated the application of information theory to studying the importance of these residues to structure and function[91]. The sequence entropy can be calculated from the probability distribution of allowed amino acids substitutions at each residue[108–110]. Using simulations of evolution on fitness landscapes, Voigt et al. predicted that beneficial mutations are found by directed evolution at amino acids that are largely uncoupled to other sites (Figure 4.9)[44]. To test this prediction, they compared the calculated site entropies with mutations found from previous evolution experiments on subtilisin E and T4 lysozyme. The sequence space considered in the subtilisin E computation was enormous: 10^{343} amino acid combinations (274 residues). Seven out of the nine mutations that improved the thermostability of subtilisin E occur at positions computed to be highly tolerant. Mutations that improved activity in organic solvent similarly occurred at high-entropy positions. This calculation may be used to determine the positions where improvement will likely be found in an evolution experiment.

4.3.2
Recombination

Another approach to creating genetic diversity is based on DNA recombination. Multiple positive variants are used to parent the next generation, which allows for recombination of beneficial mutations, elimination of deleterious mutations as well as creation of new diversity. Using recombination also has a practical advantage. More than one mutant may show improved fitness during a single screening effort. Allowing only the fittest mutant to continue to the next generation can be wasteful (Fig. 4-3). For highly non-additive (rugged) fitness landscapes, recombining positive mutations is less certain to be beneficial because combining individually good mutations can have deleterious effects. In this discussion, we focus on the experimental techniques for recombination and describe the theoretical basis for the optimal parameters.

4.3.2.1
In Vitro Recombination

In vitro recombination of DNA, often referred to as DNA shuffling, was introduced by Stemmer for evolutionary protein design[36, 37]. The method is based on recursive PCR, which allows for whole gene synthesis from several DNA fragments[93]. As outlined in Fig. 4-4, one or several parental genes are cut by enzymatic digestion using the endonuclease DNase I in the presence of Mg^{2+}. This generates overlapping DNA fragments that are randomly distributed over the gene. The isolated DNA fragments are then reassembled in a PCR-like reaction with denaturation, annealing, and extension steps, during which recombination occurs through the reannealing of DNA fragments from different parents.

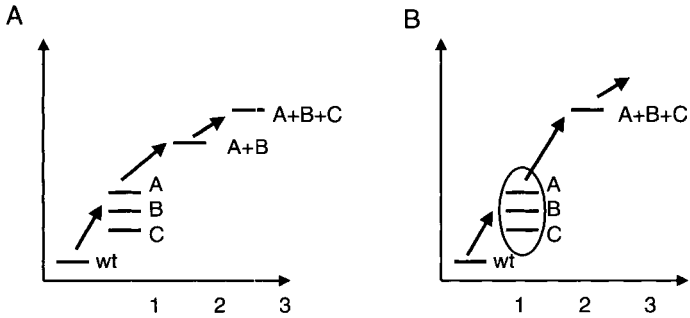

Figure 4-3. Comparison of the progress of evolution for a random mutagenesis approach (A) where the best mutant is used as parent for the next cycle of mutagenesis and screening and a DNA-recombination approach (B) where several improved mutants are used as parents for the next generations. Not shown here is any additional screening cost associated with finding several improved variants in each generation.

Diversity is created by combining parental mutations and random point mutations which are introduced at a rate of about 0.7 % because of the intrinsic error rate of *Taq* DNA polymerase[37]. A high error rate, however, can mask the relationship between evolved phenotype and combined parental mutations. Using different DNA polymerases and substituting Mg^{2+} with Mn^{2+} during DNase I digestion reduces the mutation rate to as little as 0.05 %[61]. A high-fidelity protocol is also important if recombination is used to distinguish between functional and non-functional mutations or for structure-function studies of evolved sequences[111].

In normal PCR, recombination can also occur at a low rate. This is caused by incomplete extension of primer during the extension cycle and annealing to a different template[112–114]. Increasing the recombination efficiency of incomplete primer extension motivated development of the staggered extension process (StEP)[115]. The basis of StEP is repeated switching of the template caused by fast annealing and extension cycles (Fig. 4-5). During each cycle, the growing oligonucleotide can randomly anneal to different templates and thereby combine information from different parents. A further method for *in vitro* DNA recombination is the random-priming method (RPR)[38], which involves production of gene fragments by annealing and extension of random-sequence primers. The fragments are reassembled as in the Stemmer procedure.

StEP, RPR and Stemmer's method were compared based on their recombination efficiency of truncated GFP genes[116]. The Stemmer method using small fragments (<100 bp) and StEP yielded the highest recombination efficiencies. However, the efficiencies may differ from gene to gene and are highly dependent on the experimental conditions chosen. Detailed protocols for RPR, StEP and the Stemmer method are given in[15].

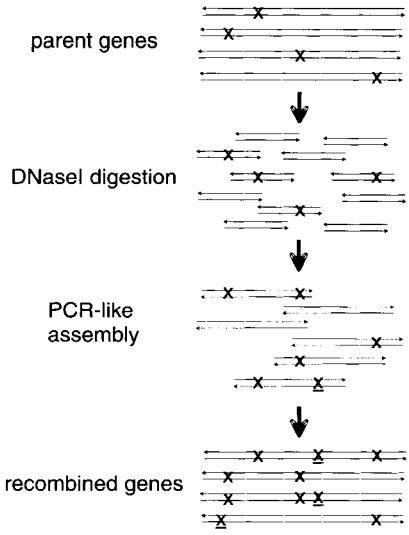

Figure 4-4. *In vitro* recombination by DNA shuffling as described by Stemmer[37]. Parent genes carrying mutations (indicated by X) are digested with DNase I and randomly reassembled in a cyclic PCR-like reaction to yield a library of recombined genes. New point mutations are introduced (indicated by underlined X) during the reassembly reaction.

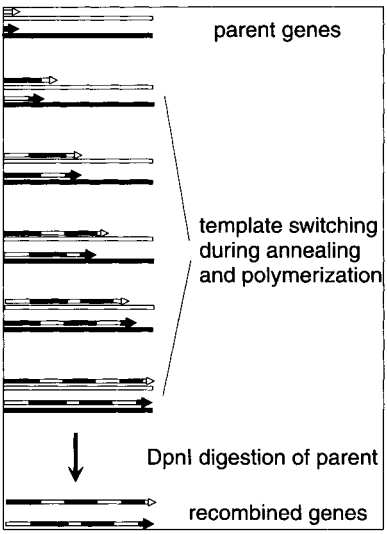

Figure 4-5. *In vitro* recombination by the staggered extension process (StEP) [115]. Only one primer and single strands from two parents are shown. Fast annealing and extension cycles during PCR of the parent genes cause template switching of the extending strand. After full-sized recombined genes are synthesized, parent genes are removed by treatment with DpnI.

It is possible to recombine any number of parent genes with the available methods, which raises the question of what is the optimal number. Similar to determining the optimal mutation rate for random mutagenesis, the answer will depend on the number of screened mutants and the additivity of the combined mutations. It could be advantageous to screen all the permutations of mutations from the parents. Assuming independent and additive recombination, the probability P_d that an offspring has d mutations is given by

$$P_d = \frac{T!}{(T-d)!d!}\left(\frac{1}{M}\right)^d\left(\frac{M-1}{M}\right)^{T-d}$$

where M is the number of sequences and T is the total number of mutations [117]. Unfortunately, only 25% of the sequences in the recombination library have novel combinations of mutations because there is a statistical disadvantage for the presence of new combinations of mutations. The probability of creating a mutant that contains all mutations is only $(1/M)^T$. If all mutations from four parents (each having two mutations) are recombined, the probability of creating an offspring gene with all the parental mutations is only 1.5×10^{-5}. This probability decreases rapidly as the number of mutations and parent genes increase. The probability is further reduced if recombination is accompanied by a high error rate. It is clear that recombination of large pools of sequences quickly reaches the throughput limitation for available screening or selection methods if the mutant containing all mutations needs to be sampled.

One strategy for reducing the screening requirements is to divide the recombination experiment into multiple generations [117]. The following example demonstrates the advantage of this procedure when the goal is to combine eight mutations onto a single gene from four double-mutant parents. The experiment is divided such that

two libraries are created using two parents each. In each library a mutant is created that contains all four mutations of its parents. The probability of creating this quadruple mutant is $p = (1/2)^4 = 1/16$. All recombinant mutants at this step can be sampled by screening only 32 clones (some oversampling will be required). If the quadruple mutants from each library are then recombined to create a mutant with all eight mutations, the probability of creating it is $(1/2)^8$. In sum, screening fewer than 300 clones (16 + 16 + 256) would be sufficient, whereas the simultaneous recombination of all four mutants to create the same mutant in one recombination experiment would require screening about 65 000 clones. The success of this procedure also relies on the additivity of the mutations. If some of the mutations are non-additive, then combining all mutations is not guaranteed to be optimal. In addition, not all combinations of mutations are screened. If a particular double or triple mutant is the fittest, it may not be found (see pooling strategies, Sect. 4.2.3). Note that if the mutations to be combined were discovered in previous generations, then it is likely that they are relatively additive (or uncoupled, see Mutagenesis Hot-Spots, Sect. 3.1.3).

All of the recombination methods described above require considerable sequence identity among the parents for crossovers to occur. For example, in the Stemmer method, the fragments require a minimum nucleotide sequence identity to reanneal and form an offspring gene. Non-homologous recombination methods seek to remove the sequence identity restriction from the recombination process. Ostermeier et al.[118, 119] describe a method based on generation of N- or C-terminal fragment libraries of two genes by progressive truncation of the coding sequences with exonuclease III followed by ligation of the products to make a single-crossover hybrid library. An intrinsic problem of this approach is that random ligation of two DNA fragments results in two-thirds out-of-frame sequences, yielding mostly non-functional products. In addition, recombination of more than two parents is hard to realize. Another approach to create a mutant enzyme library is based on the permutation of modules or secondary structure units[120, 121]. It is not yet known to what extent non-homologous recombination generates *useful* genetic diversity, rich in improved functions.

4.3.2.2
In vivo Recombination

In vivo homologous DNA recombination mechanisms are known for various host cells such as *E. coli*[39] or *Saccharomyces cerevisiae*[40] and have long been applied in protein engineering purposes, for example to shuffle mammalian P-450 substrate specificities[95]. Because of its simplicity, it is an attractive tool for directed evolution of enzymes[122, 123]. The most common system is based on the ability of *S. cerevisiae* to rescue plasmids with a double-bond break by intermolecular homology-dependent recombination[124]. A plasmid is cut by restriction enzymes and transformed into yeast together with different genes or fragments thereof. Recombination at homologous positions on both sites of the gap and within homologous regions of the fragments yields a functional, self-replicating circular plasmid. The reconstitution of

the functional plasmid allows for easy detection of recombination events based on the selection marker of the plasmid. Besides its simplicity, an important feature of *in vivo* recombination is that additional point mutations are extremely rare because of the high fidelity recombination mechanism in yeast. This is particularly advantageous when the aim is solely to recombine positive mutations or eliminate deleterious and neutral ones. *In vivo* methods are unlikely, however, to generate as many crossovers as *in vitro* methods.

Volkov et al. described a hybrid *in vitro-in vivo* recombination method involving formation of a heteroduplex between two homologous sequences *in vitro* and transformation into bacterial cells[125]. Mismatches present in the heteroduplex are randomly repaired by the host cell, creating recombinant sequences composed of the elements of each parent. This approach may be particularly useful in recombining large genes or entire operons.

4.3.2.3
Family Shuffling

Recombination of homologous parent genes, referred to as "family shuffling" or "molecular breeding," introduces an additional dimension to creation of molecular diversity for directed evolution[126]. While random point mutagenesis and local recombination explores only the sequence space covered by neighboring mutants, and focused mutagenesis covers only a small fraction of the whole sequence space of the protein, family shuffling explores more distant regions of the sequence space with a lower sampling density. Since the diversity is usually created by a combination of mutations from parents that were previously selected in nature to be functional, it is suggested that family shuffling provides functional diversity that could accelerate evolution[126].

Several recent studies attest to the power of this approach[126–131]. Using a family of four genes from different species, a comparison to single-gene recombination suggested an evolutionary advantage for family shuffling[126]. Recombination of four cephalosporinase genes (57 %–82 % identity in amino acid sequence) and screening of 5×10^4 clones yielded a mutant with a 540-fold increased moxalactam resistance compared to wild-type. The mutant that was generated by family shuffling was an offspring of three out of the four parent sequences and contained 33 new amino acid substitutions. The large number of new point mutations raises the question of how so many could be tolerated. It is unclear whether these mutations contribute to the improved β-lactamase function.

An important characteristic of the family shuffling approach is that the diversity changes after each generation. During early generations, many different combinations of mutations are tested, and the best combination becomes fixed during subsequent generations. After fixation, new diversity is created only by additional random point mutations inherent to the recombination method. Thus, the most promising distant regions in sequence space are explored by recombination, followed by step-wise mutagenesis towards the fitness optimum. This hybrid methodology might take more than the maximum of four generations of recombina-

tion that have been used so far in family shuffling experiments [129]. Based on studies of genetic algorithms it was also proposed that the recombination process with additional mutations provides a powerful method for finding higher fitnesses [132].

Family shuffling should gain increasing importance with the greater availability of homologous genes as a result of the rapid accumulation of new sequences in public databases. The restrictive licensing policies for the known recombination technologies might be limiting the commercial use of recombination methods at the moment. However, this situation and technical limitations of the existing methods are stimulating the development of new and improved recombination methods.

4.4 Finding Improved Enzymes: Screening and Selection

Given a thoughtful strategy to generate a mutant library, developing a method to detect positive mutants is probably the single most important step determining success (or failure) of directed evolution. Screening refers to a qualitative or quantitative assay of each single clone or few pooled clones of a mutant library, whereas selection refers to methods that enrich positives in a pool of all members of a mutant library or, even better, allow growth only of desired variants (extensively reviewed in [133–135]). In directed evolution, diversity is created on the genotype (DNA) level, whereas screening and selection acts on the phenotype (protein) level. A physical link between the genotype and phenotype is required because direct amino acid sequencing of positive mutants is not feasible and because DNA or RNA is required for replication of the desired mutants as well as for introduction of diversity in subsequent cycles of evolution. For the evolution of enzymes, the most simple, versatile and therefore most commonly used approach to couple genotype and phenotype employs recombinant cells. Genes are introduced (transformed) into cells and translated into proteins by the cell's natural transcription-translation machinery. For organisms such as *E. coli*, *Bacillus subtilis*, and *S. cerevisiae*, efficient transformation protocols are available that allow for the production of reasonably sized mutant libraries of 10^5–10^9 different members. In addition, recombinant protein expression is well established for those organisms, making them the current preferred choices for directed evolution experiments. In this section, we focus on general principles for searching mutant libraries and discuss important characteristics of available systems such as throughput, error level and versatility. We also discuss theoretical approaches to determining the required screening effort, analyzing the immense amount of data that are generated during the screening step and the theoretical advantages of different screening strategies.

4.4.1
You Get What You Screen For

The first rule of directed evolution is "you get what you screen for." The importance of establishing appropriate screening or selection methods cannot be overestimated. Conditions used for screening or selection should be as close as possible to the conditions where the enzyme is going to be applied. This includes the actual substrate, its concentration, pH, buffer, salt, temperature, co-solvents, and any other conditions that affect the enzyme behavior. For example, the screen can be established conveniently using an artificial substrate, the enzymatic products of which produce color or fluorescence. However, optimizing an enzyme on an artificial substrate bears the risk that gains in performance will disappear on the desired substrate. This is true for virtually all enzyme behaviors. If compromises in reaction conditions or substrates cannot be avoided, the risk of obtaining undesired solutions during several rounds of directed evolution can be reduced by using a secondary screen under more "real" reaction conditions or by testing the chosen mutant(s) with the actual substrate after each cycle of directed evolution[99]. For *in vivo* selection, which uses the indirect measurement of cell growth as an indicator of enzyme performance, one has to be aware of the immense flexibility (and creativity) of living organisms to circumvent selection pressures by inventing new solutions unrelated to the enzyme and its property we wish to optimize. Many such examples are well known from studies of metabolic acquisitions through laboratory selection, where strong selective pressure uncovered solutions to biochemical blocks[32, 33, 136]. Directed evolution experiments using combinatorial mutant libraries have also found complementation that was caused by activating a novel gene locus rather than by the mutated enzyme[137]. Starvation under selection conditions may induce low-fidelity polymerases and speed up the evolution of new solutions[138, 139].

4.4.2
Screening Strategies

Screening methods allow enzyme behavior to be measured independently of biological function. Novel enzyme properties can be explored, such as activity or stability in unnatural environments (e.g., extreme pH, temperatures, or organic solvents) or activity on unnatural substrates. These properties are impossible to target using *in vivo* selection methods, because of the cell's inability to survive under such biologically harsh conditions. The advantage of higher versatility and better control of the applied selection pressure comes at the cost of lower throughput (defined as the number of clones that can be tested in a given time period). Depending on the screening methods, libraries of about 10^4–10^6 clones can be screened within a few days, which is several orders of magnitude less than the 10^7–10^{11} clones that can be tested with selection methods. High throughput is usually accompanied by a high error level in the measurement, which dictates the minimum change that can be detected. On one hand, the consequence of low throughput is missing rare beneficial mutants because they are not sampled. On the

other hand, mutants will also be missed if the method cannot resolve the small fitness difference between a mutant and the parent (Fig. 4-6). Part of this difficulty stems from the fact that only a few mutations are made at each generation, and it is often only over multiple generations that large fitness improvements are observed.

It is important to take both the throughput and the error level of the assay into account when setting up a screen. The intrinsic error level of a screen can be tested by screening a number of wild-type clones, which allows an estimate to be taken of the minimal change that can be resolved. Fig. 4-7 shows typical statistics of a screen using a 96-well microtiter plate pH-indicator assay, specifically applied for the evolution of a hydantoinase[65]. From the standard deviation (in this case, 5%) and maximal deviation (20%), one can estimate that mutants differing in more than 50% activity can be detected with high confidence. Thus, this screening method is suitable for detecting small improvements in activity in a mutant library.

4.4.2.1
Low-Throughput Screening

Screening in 96- or 384-well plate formats allows precise fitness measurements. The accuracy of the detection system, kinetic assays (in contrast to end point assays), the possibility to normalize activity values (e.g., using measured cell concentrations), and better control over cell growth and protein expression contribute to the improved

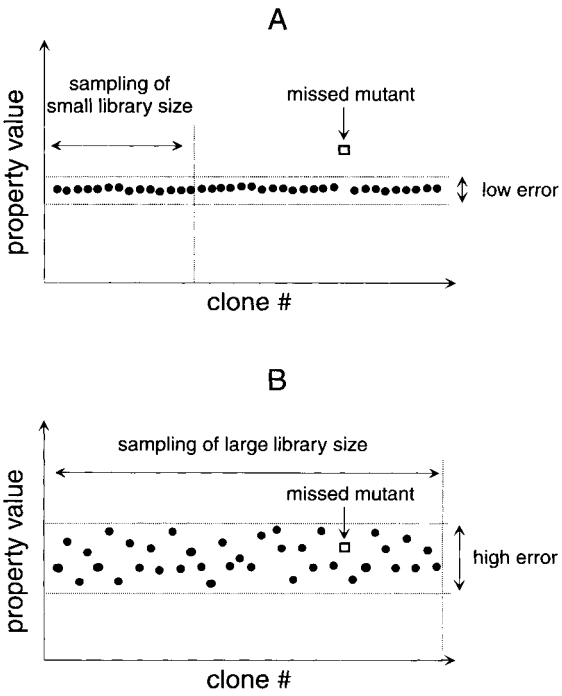

Figure 4-6. Throughput (sampling size) versus error level of a screen. Low error level and small sampling size (A) might miss the best mutant because it is too rare to be sampled. Large sampling size and high error level (B) might miss the mutant because it cannot be distinguished from wild-type background even though it was sampled.

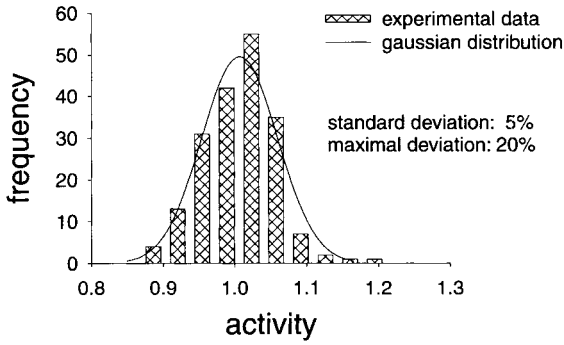

Figure 4-7. Statistics of a typical screen using a 96-well microtiter plate pH-indicator assay which was applied for the evolution of a hydantoinase[65]. Total # clones = 192. The experimental data roughly follow a Gaussian distribution.

accuracy of microtiter plate-based screening systems compared to colony-based screens. Semi-quantitative visual screens are usually based on fluorescence[140–142], color formation[16, 143], or formation of clear zones (halos)[144, 145] of colonies grown on agar plates (often filter membranes). The time-consuming process of gridding transformed colonies into microtiter pates is not required. Further, sample preparation steps are straightforward (and sometimes not necessary at all), which simplifies and accelerates the screening process. If coupled to digital imaging analysis, visual screens can be used for quantification[146, 147]. However, the high throughput and simplicity of these methods are often balanced by a larger error. Standard analytic systems such as HPLC, GC or capillary electrophoresis allow very sensitive and precise measurement and are highly versatile. However, throughput is very limited to fewer than 10^3 samples per week. This is too low for efficient screening of most mutant enzyme libraries. Further developments, such as integration of HPLC, GC, or capillary electrophoresis into automatic liquid handling systems, coupling of mass detection systems, and parallel separations will increase the throughput.

4.4.2.2
High-Throughput Screening

Complete automatic systems are now available that can screen up to a thousand 96-well plates per day (about 10^6 samples per week). Systems have been developed to increase the density of the plates (number of samples per area) by reducing the required sample volume[148, 149]. This reduces the cost of screening each mutant and increases the throughput of the assays. In screening enzyme libraries, the throughput seems to be more limited by the required step to transfer single clones from agar plates into the arrays of microtiter plates or silicon wafers rather than by the assay step. Robotic systems are sometimes used to speed up this transfer. An alternative to this step might be a dilution that adjusts cell density to one cell within a certain liquid volume, which could be transferred into adequate plates (or onto silicon wafers) much faster. Although the theoretical throughput would be increased, the statistical problem arises that a huge fraction of the transferred volume would be empty or contain more than a single cell. Other ultra-high-throughput systems that

can directly analyze single cells or single proteins do not require a transfer step and thus have a potentially higher throughput. Confocal fluorescence coincidence analysis (CFCS) can analyze up to 10^7 single molecules or cells per week[150]. Although the reported sensitivity ($<10^{-15}$ mM can be detected by regular FCS[151]) and throughput are impressive, applications for directed evolution of enzymes have not yet been reported. A fluorescence-activated cell sorter (FACS) can be used to analyze as many as 10^9 mutants per week. It is currently the only available screening tool with a sufficiently high throughput to exhaustively screen mutant libraries for all possible double mutants in less than a week. So far, most reported applications of evolutionary protein design using FACS to screen and sort mutant libraries have been for binding molecules[68, 92]. Joo et al.[146] describe a plate-based fluorescence digital imaging screen with a throughput of $\sim 10^5$ clones per day.

4.4.2.3
Choosing Low versus High Throughput

In this section, we will discuss the critical issues in deciding the balance between throughput and accuracy in the screen. A minimal screening requirement can be roughly estimated from the frequency of positive mutants found either in earlier rounds of directed evolution or from results reported in literature. The frequency of positive mutants for different enzymes and different properties varies, but is usually found in the range of about one out of 10^2 to 10^5 mutants (Table 4-2). However, it should be noted that the frequency of positive mutants will strongly depend on the fitness of the parent the property, and the strategy chosen to create the mutant library.

A theoretical approach to determine the required number of screened mutants is based on the landscape paradigm. Following this paradigm, several studies have shown that, when the landscape is additive, the number of mutants that need to be screened in order to find fitness improvements increases linearly as the wild-type sequence increases in fitness[29, 158]. However, as the landscape ruggedness increases, the number of fitter neighbors decreases more rapidly as the sequence becomes optimized[30, 159, 160]. Thus, in order to discover improved mutants, the number of mutants screened has to increase more rapidly on rugged landscapes. This implies that a protein that is tolerant (corresponding to a smooth landscape) can undergo more rounds of mutation and improvement.

There is a tradeoff between generating large libraries for a few generations and generating small libraries for many generations. In other words, if the total number of mutants that can be screened is fixed, what is the optimal number of generations? While the improvement in fitness increases with the size of the screening library, the benefit of accumulating stepwise positive mutations over multiple generations is compromised. Both experimental and theoretical studies have suggested that the best method may be short, adaptive walks utilizing small libraries[14, 161]. Husimi and Aita further studied the effect of the screening cost on the optimal search strategy[78]. They found that screening multiple generations of small libraries has the advantage of evolving more rapidly; however, it has a greater potential of being

Table 4-2. Frequencies of positive mutants found during directed evolution studies. Calculation is based on reported mutants and might not represent all positive mutants. Actual frequencies may be higher.

Frequency of positive mutants (tested/positive)	Evolved property / mutagenesis procedure / test	Reference
3×10^{-3} (1500/5)	Thermostability / PCR / screen	[152]
5×10^{-4} (2000/1)	Thermostability / family shuffling / screen	[128]
2×10^{-5} (1.5×10^{6}/34)	Thermostability / chemical / selection	[153]
3×10^{-5} (6.4×10^{4}/2)	Thermal and oxidative stability / PCR / screen	[123]
3×10^{-3} (10^{3}/3)	Activity in organic solvent / PCR / screen	[99]
2×10^{-5} (2×10^{6}/35)	Activity at elevated temperature / PCR / screen	[154]
4×10^{-5} (2×10^{5}/7)	Activity / cassette mutagenesis / selection	[88]
1×10^{-2} (10^{4}/124)	Activity / family shuffling / screen	[129]
3×10^{-3} (300/1)	Activity in organic solvent / PCR	[155]
2×10^{-3} (1.7×10^{3}/4)	Activity / family shuffling	[130]
8×10^{-5} (1.2×10^{4}/1)	Functional expression / PCR / screen	[156]
1×10^{-2} (10^{3}/12)	Enantioselectivity / PCR /screen	[157]
1×10^{-3} (750/1)	Enantioselectivity / mutator strain / screen	[49]
4×10^{-2} (150/6)	Substrate specificity / focused mutagenesis / screen	[87]

trapped in a local optimum. If the cost of screening a mutant is high, then the walk should tend towards many generations of small libraries, whereas, if it is low, it should tend towards fewer generations of large libraries. A powerful strategy for balancing the limitations of throughput and sensitivity is tiered screening (Fig. 4-8). In this method, a series of screening or selection methods with decreasing throughput and increasing accuracy are combined. This strategy has been used successfully for the evolution of subtilisin towards a combination of properties[127] and for the evolution of an esterase towards increased enantioselectivity[49].

A computational method, such as the entropy calculation presented in Sect. 4.3.1.3, can be used to eliminate the portions of sequence space where improvement is unlikely (Figure 4.9)[82]. Pre-screening drastically reduces the number of mutants that have to be experimentally screened.

Another approach to increasing throughput is to use a pooling strategy[162–164]. This methodology is conceptually equivalent to the recombination strategy presented in Section 4.3.2, in which the recombination load is subdivided into multiple generations, thus reducing the required screening effort. Most screens, however, are not sufficiently sensitive to use a pooling strategy to find small improvements.

4.4.2.4
Analyzing the Mutant Fitness Distribution

During the screening, a large amount of fitness data is generated, but only the fitness information of the improved mutants is used to continue to the next round of evolution. The large ensemble of less fit mutants provides a view of the local fitness landscape. By analyzing these data, certain statistical landscape parameters can be

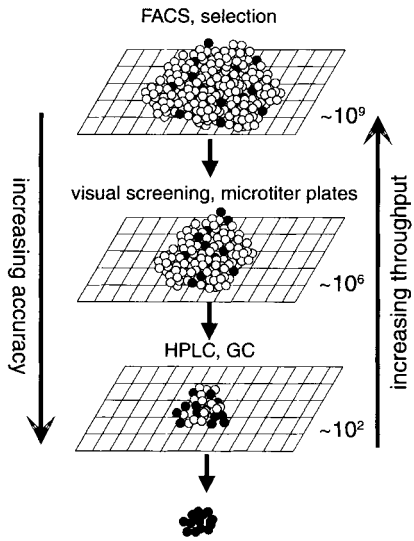

Figure 4-8. Strategy of tiered screening. Series of screening or selection methods with decreasing throughput and increasing accuracy are combined.

deduced, such as the fitness landscape ruggedness, which can then be used to guide in setting evolutionary parameters. In this analysis, sequencing is time-consuming and expensive, so a sequence cannot be assigned to each measured fitness. The lack of sequencing data means that only the probability distribution of mutant fitnesses can be analyzed. In this section, we discuss some methods that have been developed to extract useful information from the mutant fitness distribution.

Several theoretical approaches based on the additivity of mutations have been proposed to analyze the screening data. Urabe and coworkers developed a model that captures additive and non-additive mutational effects in directed evolution and fit their theoretical model to the experimental fitness distribution of catalase I. By investigating the degree of non-additivity of specific properties, they tuned the parameters of the experiment to suit the fitness landscape [165]. In a similar approach, Aita and Husimi proposed that the additive model can be applied to give a rough estimate for the Hamming distance from the wild-type to the optimum, the fitness slope near the wild-type, and the height of the optimum [158]. They calculated the expected fitness distribution and compared this to experimental data produced by the mutagenesis of a region of *E. coli* lac promoter. Based on a fit between the theoretical and experimental distributions, they estimated that the Hamming distance between the wild-type lac promoter and the optimum is 7–10 nucleotide substitutions and the activity could be improved 100- to 1000-fold.

Mean-field theory can be used to predict the effects of mutation rate, landscape ruggedness, and parental fitness on the moments of the mutant fitness distribution [79]. In this analysis, only the portion of the mutant distribution that is not dead (zero fitness) or parent (unmutated) is considered. The mutant effects are averaged over the transition probabilities. In order to obtain the fitness distribution, two sets of probabilities are required: (1) the probabilities $P_i(a)$ that a particular amino acid identity a exists at a residue i, and (2) the transition probabilities that one amino acid

will mutate into another, $q_{a\to b}$. The probabilities $P_i(a)$ can be determined through a mean-field approach and the probabilities $q_{a\to b}$ are calculated based on the genetic code[110, 166–168]. Using the mean-field solution, the change in the mutant fitness distribution is captured as the sequence ascends the fitness landscape. By increasing the coupling interactions between residues, the effect of the landscape ruggedness on the moments is calculated. As the fitness of the wild-type increases, the first and second moments increase (Fig. 4-9). In other words, as the sequence ascends the fitness landscape, the mutant distribution spreads out (diffuses) and becomes skewed towards less-fit mutants (drifts). In addition, the dependence of the moments on mutation rate can be predicted. As the mutation rate increases, both the drift and the diffusion of mutants from the parent increases. Because rugged landscapes have less correlation between parent and offspring fitnesses, the drift-diffusion effect becomes exaggerated as the coupling between residues increases. Through this approach, it may be possible to model the mutant fitness distribution to experimentally obtain statistical parameters that describe the fitness landscape.

4.4.3
Selection and Methods to Link Genotype with Phenotype

The advantage of high throughput, together with minimal experimental and technical effort, makes selection an attractive tool for *in vitro* evolution. The most commonly used *in vivo* selection approach links a targeted enzyme property to cell growth through their contribution to resistance[36, 37, 88, 126, 169] or complementation of auxotrophy or genetic defects that block the metabolism of a host strain[62, 89, 90, 100]. Most of these systems allow cells with improved enzymatic properties to grow, while cells with wild-type properties do not. Such systems are especially powerful if the selection pressure can be adjusted as the evolution progresses, for example, by increasing antibiotic concentrations[36, 37], decreasing substrate concentrations or changing the enzyme expression level[62].

If the growth of cells with wild-type activity cannot be prevented and positive mutants contribute only to an increased growth rate, cells with improved variants are usually enriched by continuous culture techniques, dilution series, or detection of the size of tested colonies. A problem for selection arises if enzymes are secreted into the culture medium. This problem was avoided in one case by growing cells in hollow fibers that limit cross-feeding between neighboring colonies[170]. Another limitation of *in vivo* selection methods is that selection conditions must be compatible with the requirements of the host organisms, which are often very different from conditions under which the enzyme is going to be applied. This is particularly true if the enzyme is to be used in an industrial reactor, where conditions often involve extreme temperatures and the substrates are suspended in organic solvents. It might be advantageous to use other expression hosts that grow under different reaction conditions than the commonly used organisms (*E. coli*, *S. cerevisiae* or *B. subtilis*). Thermophilic hosts with reasonable transformation efficiencies, such as *Bacillus stearothermophilus* or *Tetrahymena thermophilus*, have been used for selection for increased thermostability by growth at elevated temperatures[171–173]. Other *in vivo*

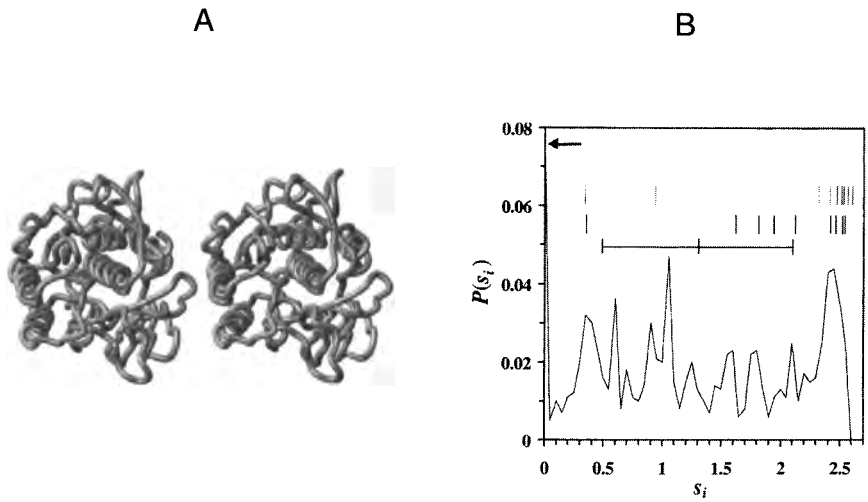

Figure 4-9. A histogram of the residue entropies for the structure of subtilisin E[82]. The entropy is a measure of the number of amino acid mutations that can be made at a residue without disturbing the structure, as determined using a model of stabilizing interactions (van der Waals, electrostatics, hydrogen-bonding, and the hydrophobic effect). When all amino acids are equally allowed at a residues, then $s_i = \ln 20 \approx 3.0$. When a single amino acid is allowed at a residue, then $s_i = 0.0$ (marked by the arrow). The connected bar in the center of the graph marks the mean and standard deviation of the histogram. The lines above this bar indicate where beneficial mutations that improved stability (top row) and activity in organic solvents (bottom row) occurred in directed evolution experiments. Most of these beneficial mutations occurred at residues that are predicted to have high entropies.

selection approaches are based on infectivity of phages[174, 175] rather than on cell growth. This method has been applied to select for proteins with improved thermostability[153] and stability against proteolysis[176]. Unfortunately, many of the targeted enzyme properties and catalyzed reactions cannot be linked to cell growth or infectivity of phages. Even if selectable traits exist, it remains a tedious task to guarantee that the targeted enzyme property and not other factors such as substrate uptake or other metabolic steps is limiting for growth.

Selections also face difficulties in controlling biological complexity. New *in vitro* selection methods might reduce some of the limitations imposed by biological complexity of living cells[5, 19, 24]. In addition, much larger mutant libraries can be searched by *in vitro* selection methods if they are used together with *in vitro* genotype-phenotype coupling systems. However, such methods often select enzymes based on single turnover events[177, 178], binding of transition state analogs[150, 179, 180] or suicide inhibitors[181] and therefore do not necessarily reflect enzyme properties of highly active catalysts.

Another *in vivo* approach to couple the genetic information with a screenable or selectable phenotype is phage display, which has been extensively reviewed elsewhere[19, 21, 23, 182, 183]. The most commonly used approach is to fuse the mutage-

nized target genes to a coat protein gene of filamentous bacteriophages. After transformation of bacteria with the recombinant DNA, bacteriophages are assembled that display the protein of interest on their surface fused to the coat protein and carry the genetic information for the displayed enzyme in the DNA. The typical diversity of a library produced by phage display is high: 10^7–10^{11} different sequences. However, it is difficult to screen for properties other than binding[184]. Furthermore, the folding of displayed proteins occurs in the periplasm of the bacteria, which is often problematic for the functional display of cytoplasmic enzymes. In addition, large and multimeric enzymes are difficult to display. However, a few examples exist where some of these limitations have been overcome, such as alkaline phosphatase[185], β-lactamase[186], glutathione transferase[181] and penicillin G acylase, which is a 86 kDa heterodimeric enzyme[187]. Several theoretical models have been proposed to capture the dynamics of phage display[188–191].

All *in vivo* approaches require the transformation of the mutated genes into cells, which limits the library size that can be produced to a maximum of about 10^9–10^{10} members[192]. *In vitro* approaches circumvent the transformation step by using cell-free transcription/translation systems that can produce the mutant proteins directly from the mutated genes[193]. The required link between the gene and the protein produced can be achieved with ribosome display[194] or mRNA-protein fusions[195, 196]. These elegant systems allow the generation of protein libraries that contain up to 10^{13} different members, which is a 10 000-fold expansion of accessible sequence space. Theoretically, such methods can cover all possible triple mutants of an enzyme with 300 amino acid residues if coupled to a suitable selection system. Another approach to coupling a phenotype with its genotype is based on *In vitro* transcription / translation reactions compartmentalized in water-in-oil emulsions[126, 139]. In principle, each aqueous compartment contains one single gene that is transcribed and translated into protein. In practice, however, many compartments will be empty or contain more than one gene. All these approaches require suitable screening or selection technology to make use of the larger libraries.

4.5 Applications of Directed Evolution

With directed evolution we can engineer enzyme properties rapidly and with a high probability of success. Many enzymes that have been improved by directed evolution are listed in Tab. 4-3. This powerful biocatalyst engineering strategy creates new opportunities in organic synthesis: new and improved bioconversion processes can be developed and novel compounds that are otherwise inaccessible by classical chemistry can be synthesized. In addition, the molecules created by directed evolution offer an excellent opportunity for improving our still poor understanding of sequence-structure-function relationships.

The specific applications of directed evolution that are described below focus on properties that are important for efficient enzyme production as well as on those that are of special interest for applications in organic synthesis, including enzyme

specificity, activity towards non-natural substrates, and function in non-natural environments.

4.5.1
Improving Functional Enzyme Expression and Secretion

Pharmaceutical or industrial applications of enzymes require their production at a large scale. This is usually done by overexpressing the enzyme in *E. coli*, *Bacillus sp.*, yeasts or fungi. Many enzymes, however, fail to fold properly in heterologous hosts. This is a problem particularly for membrane-bound, highly glycosylated or disulfide bond-containing eukaryotic proteins. Eukaryotic enzymes such as glycosylases, peroxidases or cytochrome P-450s will require significant improvements in functional expression to make them available in large quantities and at low cost.

Enzyme expression can be affected by mutations in the structural gene that may or may not change amino acid sequence or by mutations in regulatory elements that control expression. It was reported that horseradish peroxidase (HRP), a glycosylated heme enzyme, cannot be expressed in functional form in *E. coli* or yeast. We found, however, that we could obtain significant levels of HRP activity in the supernatant of yeast cultures by accumulating point mutations in the structural gene[156]. Furthermore, mutants expressed at high levels in *S. cerevisiae* were also better expressed in *Pichia pastoris*. Although far from sufficient for commercial enzyme production, the activity level that was achieved enabled us to carry out further generations of directed evolution to tailor the enzyme for specific applications.

Other reports show that intracellular expression of misfolded or unstable proteins can be dramatically improved by directed evolution. For example, directed evolution increased the expression of disulfide-containing antibody fragments in *E. coli* 50-fold, to reach a level of more than 0.5 g/L[212]. The expression of a wide spectrum amidase of *B. stearothermophilus* in *E. coli* was improved 23-fold by two mutations[210]. And, *in vivo* fluorescence of the green fluorescent protein was improved 45-fold by increasing its solubility and native folding in *E. coli*[140].

Secretion into the culture medium is preferred for some industrial enzymes because it can facilitate purification. This is especially true if the protein reaches high concentrations. Schellenberger's group at Genencor devised a method to select for improved subtilisin-secreting mutants in *Bacillus*[170]. Mutants secreting up to five times as much enzyme were found after one round of error-prone PCR and selection.

Directed evolution can improve functional enzyme expression and even allow expression of enzymes that are otherwise difficult to produce in recombinant systems. Results obtained by directed evolution, however, will depend on the particular system, and high expression will undoubtedly be best achieved by a combined approach of molecular biology, fermentation optimization and directed evolution.

Table 4-3. Examples of designed enzymes by directed evolution.

Target enzyme	Target function	Change effected	Approach	Organism	Reference
Kanamycin nucleotidyltransferase	Thermostability	>200-fold increase in half-life at 60–65 °C	Mutator strain + selection	*B. stearothermophilus*	[167]
Subtilisin E	Activity in organic solvents	~ 170-fold increase in 60 % dimethylformamide	Random mutagenesis + screening	*B. subtilis*	[14, 197]
β-Lactamase activity	Activity towards new substrate	32 000-fold greater resistance to cefotaxime	DNA shuffling + selection	*E. coli*	[37]
Subtilisin BPN	Stability in the absence of Ca^{2+}	1000-fold increase in half-life	Loop removed + cassette mutagenesis + screening	*B. subtilis*	[198]
p-Nitrobenzyl esterase	Activity towards pNB esters; activity in organic solvent	60- to 150-fold increase	Random mutagenesis + DNA shuffling + screening	*E. coli*	[99, 117, 199]
Thymidine kinase	Substrate specificity (gene therapy)	43-fold increase in sensitivity to gancyclovir in hamster cells	Cassette mutagenesis + selection + screening	*E. coli*	[85]
β-Galactosidase	Activity towards new substrate; substrate specificity	66-fold increased activity; 1000-fold increase in substrate specificity	DNA shuffling + screening	*E. coli*	[16]
O6-alkylguanine-DNA alkyltransferase	Protection against alkylating agents (gene therapy)	10-fold increased protection against toxic methylating agent	Cassette mutagenesis + selection	*E. coli*	[84]
Arsenate detoxification pathway	Arsenic resistance	12-fold increased rate of arsenate reduction	DNA shuffling + selection	*E. coli*	[200]
Aminoacyl-tRNA synthetase	Aminoacylation of a modified tRNA	55-fold increase in activity	DNA shuffling + selection	*E. coli*	[11]
Aspartate aminotransferase	Activity towards β-branched amino and 2-oxo acids	10^5 increase	DNA shuffling + selection	*E. coli*	[62]

Table 4-3. (cont.)

Target enzyme	Target function	Change effected	Approach	Organism	Reference
Lipase	Wash performance	Improved performance in one-cycle wash	Mutagenesis + *in vivo* recombination + screening	*S. cerevisiae*	[201]
Lipase	Enantioselectivity in hydrolysis of *p*-nitrophenyl 2-methyldecanoate	Increase in enantiomeric excess from 2% to 81%	Random mutagenesis + screening	*E. coli*	[157]
Lipases	Activity towards long-chain *p*-nitrophenyl esters	3-fold increase	*In vivo* recombination of homologous genes + screening	*E. coli*	[122]
pNB esterase	Thermostability	14 °C increase in T_m + increased activity at all temperatures	Random mutagenesis + DNA shuffling + screening	*E. coli*	[152]
Esterase	Enantioselectivity of hydrolysis of a sterically hindered 3-hydroxy ester	Increase in enantiomeric excess from 0% to 25%	Mutator strain + screening	*E. coli*	[49]
Subtilisin E	Thermostability	17 °C increase in T_{opt} + increased activity at all temperatures	Random mutagenesis + DNA shuffling + screening	*B. subtilis*	[202]
Subtilisin E	Thermostability	50 × increase in half-life at 65 °C	DNA shuffling + screening	*B. subtilis*	[115]
B. lentus subtilisin	Expression level (total activity of secreted enzyme)	50% increase	Random mutagenesis + enrichment in hollow fibers	*B. subtilis*	[170]
Subtilisin BPN	Activity at 10 °C	2-fold increase	Chemical mutagenesis + screening	*E. coli*	[203]
3-Isopropylmalate dehydrogenase	Thermostability	3.4-fold increase in activity at 70 °C	Spontaneous mutations + selection	*Th. thermophilus*	[204]
Cephalosporinases	Activity towards moxalactam	270- to 540-fold increased resistance	DNA "family" shuffling + selection	*E. coli*	[126]

Chorismate mutase	Conversion to monomeric enzyme (solubility)	Functional monomeric enzyme	Oligonucleotide directed codon mutagenesis + selection	*E. coli*	[83]
Biphenyl dioxygenases	Degradation of polychlorinated biphenyls (PCBs)	Gained activity towards substrates poorly degraded by native enzymes; improved activity towards various substrates	DNA "family" shuffling + screening	*E. coli*	[131]
FLP recombinase	*In vivo* recombination efficiency at elevated temperatures in *E.coli* and mammalian cells; *in vitro* thermostability	Improved recombination efficiency in *E. coli* and mammalian cells	Random mutagenesis + DNA shuffling + screening	*E. coli*	[154]
EcoRV endonuclease	Extend recognition site	Becomes 10 bp cutter	Targeted random mutagenesis + screening	*E. coli*	[87]
Cytochrome P450cam	Increased activity in peroxide shunt pathway, towards naphthalene	5- to 20-fold increase	Random mutagenesis + step shuffling + screening	*E. coli*	[142]
Aspartate aminotransferase	Substrate specificity	2.1×10^6-fold increase in cat. efficiency towards valine	DNA shuffling + selection	*E. coli*	[100]
Glutathione transferase	Substrate specificity	Found range of specificities	DNA "family" shuffling + screening	*E. coli*	[205]
Peroxidase	Stability to peroxide, thermostability	110 × greater thermal stability, 2.8 × oxidative stability	Random mutagenesis + *in vivo* recombination + site-directed mutants + screening	*Yeast*	[123]
β-Glucuronidase	Retention of function after glutaraldehyde cross-linking	More resistant to glutaraldehyde and formaldehyde	Random mutagenesis + DNA shuffling + screening	*E. coli*	[206]
Subtilisin S41	Improved thermostability	100-fold increase in half-life	Random mutagenesis + saturation mutagenesis + screening	*B. subtilis*	[64]

Table 4-3. (cont.)

Target enzyme	Target function	Change effected	Approach	Organism	Reference
Hydantoinase	Enantioselectivity + total activity	Inverted enantioselectivity, 5 × increase in total activity	Random mutagenesis + saturation mutagenesis + screening	*E. coli*	[65]
Subtilisins	Various properties	Improved activity stability	DNA "family" shuffling + screening	*B. subtilis*	[127]
Esterase	Enantioselectivity	Two-fold improved E	Random mutagenesis + screening	*E. coli*	[207]
Thymidine kinase	Substrate specificity	7–44 fold improved specificity	DNA "family" shuffling + screening	*E. coli*	[129]
Catechol 2,3-dioxygenases	Thermostability	13–26 × more thermostable	DNA "family" shuffling + screening	*E. coli*	[128]
Lactate dehydrogenase	Cofactor (fructose-1,6-bisphosphate) requirement	70-fold activation without cofactor (fully active in the absence of cofactor)	DNA shuffling + screening	*E. coli*	[208]
Phytoene desaturase and lycopene cyclase	New carotenoid pathway (substrate and reaction specificity)	Production of torulene in *E.coli*	DNA "family" shuffling + screening	*E. coli*	[209]
Indole-3-glycerol-phosphate synthase	Confer new catalytic activity (phosphoribosyl anthranilate isomerase)		Rational design + DNA shuffling + selection	*E. coli*	[17]
Kanamycin nucleotidyl transferase	Thermostability	Increase 20 °C	DNA shuffling + screening/selection	*T. thermophilus*	[171]
B. stearothermophilus amidase	Increase expression in *E. Coli*	Increase expression (23 ×)	Random mutagenesis + screening	*E. coli*	[210]
Horseradish peroxidase	Increase activity/ expression in *S. cerevisiae*	Increase total activity 40 ×	Random mutagenesis + screening	*S. cereivisiae*	[156]
Cytochrome P450 BM-3	Substrate specificities	Hydroxylates indole	Saturation mutagenesis + screening	*E. coli*	[211]

4.5.2
Engineering Enzymes for Non-natural Environments

Bioconversion processes performed in organic solvents or at elevated temperatures impart such benefits as increased substrate solubility, decreased viscosity of the reaction medium, altered reaction selectivity and equilibria, higher rates, and reduced risk of microbial contamination. High thermostability also tends to translate to resistance to other denaturants and better long-term stability at lower temperatures. Most natural enzymes are poorly suited for function in organic solvents or at high temperatures, however, and their limited stability and activity in these environments can be a limiting factor for applications in organic synthesis. These properties are good targets for engineering by evolution.

Directed evolution has generated a large number of thermostabilized enzymes (see Table 4-3 for examples); there are too many reports for a comprehensive review here. We will discuss only the general picture that arises from those studies; the interested reader is referred to a recent review that deals with evolution of enzyme stability in greater detail [213].

The increase in thermostability imparted by single amino acid substitutions is usually small and is typically in the range of a 1–2 °C increase in melting temperature or optimal reaction temperature [152, 171, 173, 214–216]. Larger changes are possible, but rare. Significant changes in thermostability therefore require the accumulation of multiple substitutions, e.g., by sequential rounds of mutagenesis or recombination. This strategy has generated 20 °C and higher increases in thermostability [15, 171]. The stabilization mechanisms are consistent with those found in naturally thermophilic enzymes and include reduction of surface loop flexibility, new hydrogen bonds, altered core packing, helix stabilization and acquisition of surface salt bridges [217]. Although the mechanisms are familiar, most of the changes would have been difficult or impossible to predict.

In nature, thermophilic enzymes tend to be less active at low temperatures than their mesophilic counterparts, which in turn are less thermostable (Fig. 4-10). One popular explanation for this observation is that activity and stability make mutually exclusive demands on enzyme flexibility. Two properties coupled in this way cannot evolve independently. However, directed evolution experiments have shown that these properties *can* evolve independently [15, 152, 202]. A 17 °C increase in the melting temperature of a mesophilic esterase was achieved at the same time as catalytic efficiency was increased several fold by random mutagenesis and screening over several generations [152, 213]. A similar approach taken with mesophilic subtilisin E generated a 17 °C increase in the temperature optimum and a >200-fold increase in half-life at 65 °C [202]. The thermostable subtilisin was also more active than wild-type over the whole temperature range. Most recently, directed evolution of a psychrophilic subtilisin [15] led to a 500-fold increase in half-life at 60 °C at no cost to its activity at low temperature. The evolved enzyme is more stable than homologous mesophilic subtilisins. The stabilized enzymes contained between 7 and 13 amino acid substitutions. In the studies described above, mutants were screened simultaneously for activity and thermostability, and mutations were accepted only when

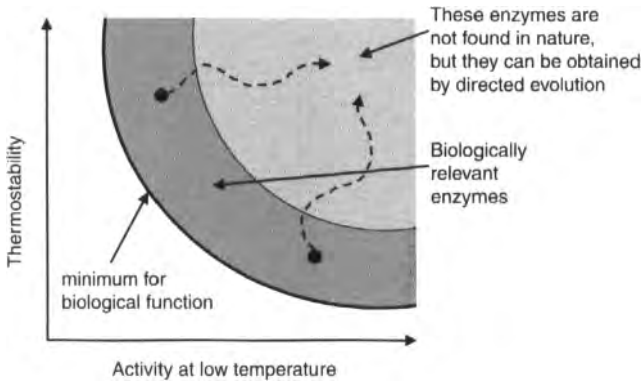

Figure 4-10. Enzymes isolated from organisms growing at different temperatures often exhibit a tradeoff between thermostability and catalytic activity measured at low temperature. Enzymes that are both highly thermostable and highly active at low temperatures are rare in nature but highly desired for various applications and can be obtained by directed evolution with relatively few mutations [184].

enhanced thermostability came at little or no cost to activity. If the selection pressure is not maintained, thermostability can easily be lost [218, 219].

Creating enzymes that are both more thermostable and more active is particularly exciting for industrial applications. In addition, these studies nicely demonstrate that behaviors of natural enzymes may not necessarily be due to physical limitations intrinsic to proteins themselves, as is often assumed. Instead they reflect what is both relevant to the organism and accessible to natural evolution [184].

Directed evolution has also been very effective for increasing enzyme activity in organic solvents [14, 99]. For example, the serine protease subtilisin can catalyze specific peptide syntheses and transesterification reactions, but organic solvents are required to drive the reaction towards synthesis. Sequential rounds of error-prone PCR and visual screening yielded a subtilisin variant with twelve amino acid substitutions that was 471 times more active than wild-type in 60% dimethylformamide (DMF) [145, 220]; this enzyme is much more effective for peptide and polymer synthesis.

The production of cephalosporin-derived antibiotics requires a deprotection step usually catalyzed by zinc in organic solvents. Since this step produces large amounts of solvent- and zinc-containing waste material, scientists at Eli Lilly were interested in using an enzyme. Classic screening identified an esterase that catalyzed the desired reaction but performed poorly in the solvents required to solubilize the substrate. Directed evolution was therefore used to try to improve the performance of the enzyme for efficient hydrolysis of an antibiotic *p*-nitrobenzyl ester intermediate in aqueous-organic solvent mixtures [99]. Four rounds of random mutagenesis by error-prone PCR and screening followed by one recombination step improved the esterase activity 50- to 60-fold in 25% DMF and yielded mutants that performed

as well in 30% DMF as the wild-type enzyme in water. None of the six mutations found in the best mutant were in direct contact with the substrate and some were as far away as 20 Å. Thus, focused mutagenesis in the substrate binding site may have overlooked important beneficial mutations.

High product concentrations are important in organic synthesis but often detrimental to enzymes. Scientist at Celgene reduced product inhibition in transaminases[221] which are valuable for the production of chiral amines or amino acids. A single round of error-prone PCR and screening of 10 000 clones revealed mutants with better product tolerance that translated to a four-fold increase in volumetric productivity for a substituted amphetamine.

4.5.3
Engineering Enzyme Specificity

Enzymes are particularly valuable for the production of enantiomerically pure compounds, as shown in examples throughout this book. However, the narrow range of substrates that some enzymes accept and the less than impressive enantioselectivities exhibited by others often frustrate attempts to develop new synthetic applications and to commercialize existing ones. Directed evolution can efficiently tune substrate specificity and catalytic efficiency towards non-natural substrates; it can also tailor enantioselectivity, as illustrated in the examples below.

4.5.3.1
Substrate Specificity

Zhang et al. evolved a fucosidase from a galactosidase[16]. Seven rounds of DNA shuffling and screening using a chromogenic fucose substrate yielded a mutant with 66-fold increase in fucosidase activity. Kinetic analysis of the purified enzyme revealed a 10- to 20-fold increase in k_{cat}/K_m for the fucose substrate and a 50-fold decrease for galactose (a total of 1000-fold increased substrate specificity for fucose).

Kumamaru et al. recombined two biphenyl dioxygenases (96% identical) and visually screened for mutants whose substrate range differed from the parents'. These mutants degraded various biphenyl compounds more efficiently and also exhibited oxygenation activity for single-ring aromatic compounds on which neither parent was active[131].

Lanio et al. reported the tailoring of restriction endonucleases *Eco*RV specificity[87]. Focused combinatorial mutagenesis was used to make variants that cleave specific DNA sequences of eight or ten base pairs rather than the six recognized by the natural enzyme. Twenty-two amino acid residues were targeted by oligonucleotide-directed mutagenesis within three different regions of the enzyme. Screening a total of only 500 colonies over three cycles of mutagenesis was sufficient to find several mutants with high activity and high specificity for AT- or GC-flanked GATATC cleavage sites.

Aspartate aminotransferase catalyzes amino group transfer between acidic amino

acids, aspartate and glutamate, and their corresponding 2-oxo acids. The wild-type activity for β-branched amino acids is barely detectable, but was dramatically increased by directed evolution[62, 100]. The aspartate aminotransferase gene derived from *E. coli* was subjected to DNA shuffling and introduced into an *E. coli* host lacking the branched-chain amino acid aminotransferase gene and therefore allowing selection by complementation with mutant aspartate aminotransferases. The stringency of the selection was increased during the progression of evolution by omitting the substrate (2-oxovaline) from the medium, shortening the incubation time and decreasing the expression level of the mutant enzymes by manipulating the construction of the plasmid. A mutant with 10^5-fold increased catalytic efficiency (k_{cat}/K_m) for β-branched amino acids and 30-fold decrease for the natural substrate was created after five cycles of shuffling and selection[62]. This mutant was further improved to yield a mutant with a remarkable 2.1×10^6-fold improved catalytic efficiency compared to wild-type[100]. Analysis of the structure of the mutant enzyme complexed with a valine analog provided detailed insight into how the mutations affected substrate binding and demonstrated the importance of cumulative effects of residues far from the active site.

The P-450 monooxygenase from *Pseudomonas putida* was evolved for efficient utilization of hydrogen peroxide in lieu of O_2 and NADH and for improved activity towards the non-natural substrate naphthalene[142]. One round of error-prone PCR and screening of about 200 000 clones by high-throughput digital image analysis[146] revealed several mutants with increased activity. Subsequent recombination of five improved mutants yielded several variants with about 20-fold improvements in naphthalene hydroxylation activity over wild-type using hydrogen peroxide as sole cofactor.

Fructose 1,6 bisphosphate (FBP) is an allosteric activator of the thermostable L-2-hydroxyacid dehydrogenase from *B. stearothermophilus*, which might be useful for the asymmetric synthesis of chiral compounds. Since FBP is quite expensive, Allen and Holbrook wished to create an FBP-independent variant[208]. Three rounds of shuffling and screening produced a mutant L-2-hydroxyacid dehydrogenase with three amino acid substitutions that is almost as active in the absence of FBP as the wild-type is in its presence.

Recently, Schmidt-Dannert et al. reported the molecular breeding of carotenoid biosynthetic pathways in *E. coli*[209]. Two different phytoene desaturases were shuffled and expressed in the context of a carotenoid biosynthetic pathway assembled from different bacterial species. Clones containing mutant desaturases were visually screened to identify new carotenoid products. One out of approximately 10 000 colonies turned pink and produced shuffled tetradehydrolycopene instead of lycopene. The new pathway was extended with a second library of shuffled lycopene cyclases. Visual screening identified a cyclase with altered substrate specificity that produced the cyclic carotenoid torulene for the first time in *E. coli*. Complementary to the strategy of creating new polyketides by mixing and matching subunits in a multi-enzyme complex[222, 223], the combination of a rational pathway assembly and directed evolution is an exciting opportunity to create libraries of otherwise inaccessible biologically active compounds.

4.5.3.2
Enantioselectivity

Matcham and Bowen were among the first to apply an evolutionary approach to improve the enantioselectivity of an enzyme for use in chiral synthesis[221]. The wild-type enzyme (an S-selective transaminase) converts a particular β-tetralone to the corresponding amine at only 65 % *ee*. By screening a mutant library of 10 000 variants in a microtiter plate-based assay, they identified 10 mutants that produced the (S)-aminotetraline with 80–94 % *ee*. Sequencing the mutants revealed positions important for enantioselectivity and, interestingly, the existence of synergistic combinations of mutations.

The lipase from *Pseudomonas aeruginosa* (PAL) catalyzes the hydrolysis of 2-methyldecanoic acid *p*-nitrophenyl ester with only 2 % *ee* in favor of the (*S*)-acid. Reetz and Jaeger used four rounds of error-prone PCR and screening on enantiomerically pure *R* and *S* substrates to generate a more enantioselective variant that produced the desired (*S*)-acid with 81 % *ee*[157]. Additional cycles of error-prone PCR in combination with saturation mutagenesis further improved the enantioselectivity of this enzyme, which hydrolyzes the 2-methyldecanoic acid *p*-nitrophenyl ester with 91 % *ee* ($E = 25.8$) in favor of the (*S*)-acid[12].

Bornscheuer et al. improved the enantioselectivity of an esterase from *Pseudomonas fluorescens*[49, 207]. In their first report, the enzyme was evolved for hydrolysis of a 3-hydroxy ester serving as a building block in epithilone synthesis[49]. Isolated plasmids obtained from a mutator strain were transferred into *E. coli* and plated onto two different kinds of agar plates. One plate contained a pH indicator which shows active clones by a color change. The other plate contained a minimal medium and a glycerol ester as the sole carbon source. Cleavage of the glycerol ester releases glycerol, which leads to growth of active cells. One clone that produced the desired enantiomer with 25 % *ee* was identified, compared to no enantioselectivity for wild-type. The screen allowed for detection of active clones, but is not sensitive to enantioselectivity; this might explain why further improvements in enantioselectivity were not reported.

A subsequent report describes the evolution of the same enzyme for the hydrolysis of 3-phenylbutyric acid resorufin ester using both a mutator strain and error-prone PCR[207]. Mutants were screened for improved enantioselectivity based on a microtiter plate assay using the optically pure R- or S-esters. Both mutagenesis methods generated first-generation mutants with higher enantioselectivity (E=6.6 and 5.8 compared to wild-type E=3.5).

Recent results show that directed evolution can also invert enzyme enantioselectivity[65]. The hydantoinase derived from *Arthrobacter sp.* shows a substrate-dependent inversion of enantioselectivity which limits its use for the production of certain L-amino acids such as L-methionine (for applications of hydantoinases in organic syntheses see Chapter 12). By accumulation of mutations through sequential rounds of error-prone PCR and saturation mutagenesis, the enantioselectivity of the hydantoinase was inverted from *ee* = 40 % for the D-enantiomer to *ee* = 20 % for the L-isomer at 30 % conversion. Only one amino acid substitution was required for the

inversion of enantioselectivity. Furthermore, mutant hydantoinases exhibiting high D-selectivity (*ee* = 90% at 30% conversion) were also found. The L-selective mutant, whose overall activity was improved 5-fold over wild-type, was co-expressed with a racemase and L-specific carbamoylase in *E. coli*. This yielded a recombinant whole-cell catalyst with an improved hydantoin converting pathway. Application of this whole-cell catalyst for the production of L-methionine resulted in >5-fold improved productivity for >90% conversion of the racemic substrate into the optically pure product.

The optimization of whole pathways by directed evolution and their introduction into recombinant whole-cell catalysts may offer the possibility of substituting complicated multi-step processes with straightforward single-pot processes. This, of course, is highly desired for industrial applications and a major advantage of biocatalysis over other competing technologies used in organic synthesis.

4.6 Conclusions

The power of directed evolution is now well documented. These methods are robust and are able to improve industrial enzymes in reasonably short times. The first laboratory-evolved enzymes are now used commercially in laundry detergents[201]; other commercial applications are on the horizon. Directed evolution may well help move biocatalysis from an "enabling tool" to a "lowest cost approach". It also offers new opportunities to engineer multi-enzyme pathways and even whole microbes[69, 224, 225], which will lead to straightforward single-pot, multi-enzyme bioconversions and new fermentation processes based on "green" resources such as glucose or inexpensive waste materials.

Sixteen years after Manfred Eigen and William Gardiner presented the basic algorithm for evolutionary molecular engineering; it is worth commenting on the conclusion of their paper:

> *"... The clones have to be addressable; the analytical methods must combine parallel processing and automatic sampling with sensitivity and speed. With such elaboration and scale, experimental biology might well become 'Big science'."*[75]

Today's tools of evolutionary engineering certainly fulfill these requirements, and directed evolution has in fact emerged as the method of choice for biocatalyst improvement. However, we are only beginning to explore the power of evolutionary design.

The most obvious limitations of these methods are still related to the tools. Screening or selection methods require significant development time. This might be reduced by the development of versatile enzyme assays that can be adapted rapidly to specific conditions. The problem will also be reduced by integrating versatile standard analytic systems such as mass spectroscopy, HPLC or capillary electrophoresis into automatic high-throughput systems.

The finite sampling capacity of most screening methods and the low versatility of

selection methods will probably remain significant limitations. This makes it difficult, if not impossible, to generate surprising new functions that require multiple simultaneous amino acid substitutions. It is clear that more "rational" approaches, based on structure/sequence comparisons or computation, will be necessary to target key amino acid positions. Other limitations of directed evolution are inherent in the current mutagenesis and recombination methods, which strongly bias the combinatorial libraries. It is not yet clear how best to create molecular diversity for evolution. What is clear is that many of these questions and limitations can and will be addressed in the near future.

The field of molecular evolution used to focus on the past and aimed to explain the existence of today's fantastic array of biological molecules. Applied molecular evolution is changing this focus to the future, by creating molecules for a biotechnology industry of unlimited opportunities.

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5
Enzyme Bioinformatics

Kay Hofmann

5.1
Introduction

Enzymes are a particular class of proteins which, through gradual developments, have been optimized extensively to catalyze a large variety of chemical reactions[1]. The primary metabolism enzymes, whose role is the "housekeeping" catalysis of metabolically important reactions, are generally optimized for robustness and high turnover rate. By contrast, the products of secondary metabolism, which comprise e.g. colorants, odorants, hormones and toxins, frequently have complex chemical structures and require biosynthetic enzymes highly optimized for stereo- and regiospecificity. A number of enzymes, many of them from microbial sources, have already proved useful for *ex vivo* applications in synthetic chemistry. The main part of this book gives an extensive overview of biosynthetic applications of enzymes currently in use. The advent of genome sequencing, both of microbes and other organisms, has lead to a sharp increase in the information available on their enzyme repertoire and metabolic pathways. It is to be expected that these additional insights will soon find their way into biocatalytic applications, leading to a broadened base of synthetically useful enzymes.

One consequence of the increased amount of raw genomic data becoming available is the requirement for bioinformatical analysis in order to extract useful information. While there is an extensive literature on bioinformatics algorithms, on protein bioinformatics in general, and on the analysis of particular protein families, there are only a few publications dealing with enzyme-specific issues of bioinformatical analysis. This chapter tries to fill a gap by specifically addressing those aspects of protein sequence analysis that are important for identifying enzymes in genomic sequences, for understanding their mode of action, and for predicting some of their properties.

The problem of understanding the mechanism of an enzyme, particularly when pertaining to the optimization of catalytic properties, is more suitably addressed by analysis of the enzyme's three-dimensional structure instead of its sequence[2, 3]. While structural analysis is occasionally considered a subtopic of bioinformatics, this

chapter will focus exclusively on aspects of sequence analysis. No matter how fast the currently initiated projects on structural genomics proceed, the availability of a protein structure will always lag considerably behind the availability of its sequence[4–7]. Thus, any piece of information that can be derived from the sequence alone will be useful in its own right. Moreover, many tasks that are commonly believed to require knowledge about an enzyme's structure, can nowadays be performed by using the sequence alone, given that the appropriate tools are used and the analysis is done properly. Examples include the identification of active site residues or the establishment of extremely distant protein relationships with sequence similarity way below 20% identical residues[8].

Since the major part of enzyme bioinformatics is based on the results of the comparison of evolutionarily related sequences, the following paragraphs will start (Sect. 5.2) with a brief survey of protein sequence comparison approaches. Comparison of multiple sequences belonging to a single family usually reveals a specific set of conserved residues. When dealing with enzymes, the nature and positioning of the resulting conservation patterns can be indicative of the enzymatic mechanism, of cofactors involved, or of other properties of that particular enzyme family. Thus, Section 5.3 will discuss the conclusions that can be drawn from this type of analysis. Section 5.4 elaborates on the "domain" concept, both in terms of structure and of sequence. Multi-domain organization of enzymes is frequently associated with multiple functionalities, which can occasionally be separated and used for overcoming undesirable regulation mechanisms or even for combinatorial biocatalysis.

As in other areas of bioinformatics, specialized databases are of crucial importance for the field of enzyme bioinformatics. Section 5.5 provides an overview of publicly accessible databases that digest and store information on enzymes, pathways and metabolites and make it available for querying. Section 5.6, which also deals with databases, puts a focus on collections of pre-classified conservation patterns characteristic of protein families and domains, both of enzymes and non-catalytic proteins. These databases have become indispensable tools for the recognition and classification of novel enzymes, a task frequently encountered when dealing with genome sequences. Section 5.7 introduces and compares a number of strategies used to mine microbial and other genome sequences for enzymes. Finally, Sect. 5.8 attemps to give an outlook on possible future developments and on the impact of bioinformatics on the identification and optimization of enzymes for biocatalytic applications.

5.2
Protein Comparison

5.2.1
Sequence Comparison *versus* Structure Comparison

It is a widely held tenet that the three-dimensional structure of a protein family is better conserved than the sequence itself. In general, there is some truth to this assumption, although the methods of measuring structural or sequence similarity

are merely operational and the results are difficult do compare. Sequence similarity is frequently expressed in terms of "% residue identity", a measure that cannot be applied to structural comparisons. Conversely, structural similarity is usually expressed by the r.m.s. distance, i.e. the root of the mean square distance of atom pairs, which in turn cannot be applied to sequences. Nevertheless, there are a number of proteins with identical or related function, whose 3D-structures look similar to the skilled eye, while there is no apparent similarity in the amino acid sequence, at least no similarity that would exceed the 'background noise' expected from comparing two random sequences[9]. This apparent superiority of structural comparison has pervaded the recent literature and has fuelled the demand for large-scale projects in structural genomics. While such projects undoubtedly have their merits, it should not be neglected that several recently introduced or improved methods of sequence analysis come very close to the sensitivity of structural comparisons. Profile- or Hidden Markov Model-based methods in particular can make use of the enzyme-specific conservation patterns discussed in Sect. 5.3, and thus are very well suited to identifying and classifying even the most distant evolutionary relationship between enzymes. A comprehensive treatment of issues in protein comparison would be beyond the scope of this chapter; the interested reader is referred to some recent reviews on this topic[10–13].

5.2.2
Substitution Matrices in Sequence Comparisons

Most sequence comparison methods, including the modern profile techniques, are based on a "dynamic programing" algorithm introduced by Smith and Waterman in 1981[14]. The method strives to find a mathematically optimal alignment from two given sequences. The scoring system used for comparing the alignment "quality" is a compromise between being a good model of biological reality and being computationally tractable. The aim is to maximize a composite score that is calculated from all positions in the alignment. The pairing of identical residues makes a positive contribution to the alignment score; the contribution of non-identical paired residues depends on their similarity as defined by a generally valid similarity table, the substitution matrix. Similar residues are associated with positive scores, while dissimilar residue pairings give a negative contribution to the alignment score. Insertions and deletions of residues in one sequence with respect to the other are allowed, but penalized. Given a proper choice of the substitution matrix and the deletion/insertion penalty, it can be assumed that the resulting mathematically optimal alignment will be close to an evolutionarily optimal one[15, 16] (see Fig. 5-1). While there are several possible ideas of what constitutes a "biologically correct" alignment[11], the context of enzyme comparison would minimally require that corresponding active site residues of the two sequences are properly aligned.

The concept of using a substitution matrix, i.e. a knowledge-derived table for judging amino acid similarities, was introduced by Dayhoff et al. in 1978[17]. Most types of currently used substitution matrices are derived from the analysis of well established alignments, by counting which types of residues are frequently substi-

```
ADE-FGA-KL        ADEFGAKL
| | | | ||        |   |  ||
A-EDF-ASKL        AEDFASKL
```

Figure 5-1. Influence of the scoring system on the alignment appearance. The left half of the figure shows an unreasonable alignment resulting from too low deletion/insertion penalties. The right half shows a better alignment, although the "% identity" score is worse.

tuted for particular amino acids[17–20]. The resulting 20 × 20 table has high positive values for identical or highly similar residue pairs, since they can be easily exchanged by evolution without significantly altering a protein's structure or function. Dissimilar residue pairs, by contrast, have negative values, since they are rarely found in homologous positions of related proteins. It is interesting to note that not all identical residue pairings have the same positive value. For example, the Trp ↔ Trp value is very high, while almost all combinations of Trp with non-Trp residues have negative scores. The most likely interpretation is that tryptophane tends to have a very specialized role in protein architecture, which can not really be fulfilled by any other amino acid. By contrast, the Ile ↔ Ile value is not nearly as high as the Trp self-score and is only marginally higher than the Ile ↔ Leu score. The likely reason is that most functions of isoleucine can also be fulfilled by other residues such as leucine.

All commonly used substitution matrices are derived from a large collection of protein alignments, containing both enzymes and non-enzymes. Thus, favorable residue groupings tend to reflect a structural compatibility rather than a functional equivalence. It would be expected that a substitution matrix derived from particular sets of enzymes would have quite different values for residues that are frequently found in active sites.

Both the inequality of residue self-matching scores, and the above mentioned influence of the gap penalties on the alignment appearance show that the "% residues identity" value is not always a good measure for judging the similarity of two sequences. First of all, this value only makes sense when based on a biologically reasonable alignment. Moreover, identical alignment positions containing tryptophane or cysteine can be considered better indicators of sequence relatedness than conserved leucines or isoleucines.

5.2.3 Profile Methods

The profile method, introduced in 1987 by Gribskov et al.[21] and improved more recently by various groups[22–25], can be considered a generalization of the Smith and Waterman method. The idea of this technique is to abandon the traditionally equal treatment of all positions in an alignment. When using profiles, it is possible to assign a specific weight, a specific substitution matrix and a specific set of gap penalties to each alignment position. The advantage of the additional degrees of freedom lies in the possibility to incorporate *a priori* knowledge into the calculation

of the alignment score. If, for example, a sequence region is known to be very important for a protein's structure or function, it can be assigned a higher weight in the calculation of the overall alignment score. Similarly, if a position is known to be part of the active site, a particular substitution matrix could be used for that region. If the structure of a protein is known, sequence stretches corresponding to solvent-exposed regions could be assigned "cheaper" gap penalties, since it is known that external loops in protein structures can accommodate deletions or insertions more easily than the structural core.

The most typical field of profile application is the alignment of a single sequence to an already established protein family. Starting from a multiple alignment of the protein family, specialized profile construction programs (Table 5-1) look for regions with high conservation, implying a greater importance for the family's structure or function, and assigns high weights to the preferred residues in these positions. Regions that already harbor gaps in the original family alignment are considered structurally variable and are assigned lower gap penalties.

A mathematically very different approach, which is formally equivalent to the generalized profile method, uses so called Hidden Markov Models (HMMs). A more

Table 5-1. Unified resource locators (URLs) for online accessible information sources mentioned in the text.

Section 2: Profile and HMM construction programs	
pftools	http://www.isrec.isb-sib.ch/ftp-server/pftools
HMMer	http://hmmer.wustl.edu
Section 5: Enzyme databases	
ENZYME	http://www.expasy.ch/enzyme
SWISS-PROT	http://www.expasy.ch/sprot
BRENDA	http://www.brenda.uni-koeln.de
KEGG	http://www.genome.ad.jp/kegg
LIGAND	http://www.genome.ad.jp/dbget/ligand.html
PDB	http://www.pdb.org
Enzymes Structures Database	http://www.biochem.ucl.ac.uk/bsm/enzymes
UM-BBD	http://umbbd.ahc.umn.edu
PROMISE	http://bmbsgi11.leeds.ac.uk/promise
MDB	http://metallo.scripps.edu
MEROPS	http://merops.iapc.bbsrc.ac.uk
ESTHER	http://www.ensam.inra.fr/cholinesterase
Section 6: Domain and motif databases	
PROSITE	http://www.expasy.ch/prosite
PFAM	http://www.sanger.ac.uk/Pfam
SMART	http://smart.embl-heidelberg.de
BLOCKS	http://www.blocks.fhcrc.org
PRINTS	http://bioinf.man.ac.uk/dbbrowser/PRINTS
INTERPRO	http://www.ebi.ac.uk/interpro
PROCAT	http://www.biochem.ucl.ac.uk/bsm/PROCAT/PROCAT.html
Section 7: Genome resources	
GOLD	http://wit.integratedgenomics.com/GOLD
COG	http://www.ncbi.nlm.nih.gov/COG
STRING	http://www.bork.embl-heidelberg.de/STRING

extensive coverage of the construction of profiles and HMMs, as well as their application in sequence comparisons, is given elsewhere[10–13].

5.2.4
Database Searches

A frequently encountered task in sequence analysis is to screen a sequence database for relatives of a given protein. In general, all sequence comparison methods that assign alignment scores, including the Smith and Waterman method and the profile method, can be used to that end. A straightforward way is to compare the query sequence (or profile) to each single sequence in the database and sort the results by their respective alignment score.

A major obstacle of this approach is the large amount of computation necessary for full dynamic programing algorithms, making these database searches very slow unless running on high-performance computers. Alternative database search methods, including the well known FASTA and BLAST programs[26–28], are substantially faster through their use of heuristical approximations. While these methods cannot guarantee to find the optimal solution, the minor trade-off in sensitivity is more than compensated for by the immense gain in speed. The heuristical methods are nowadays routinely used for database searches, sometimes combined with a true Smith and Waterman post-processing step for the highest scoring matches.

An additional problem that has to be faced when doing sequence database searches is the judgement of alignment score significance. Whenever comparing a query to every sequence in a database and sorting the results by score, it is inevitable that one database sequence will come out at the top of the list. However, this does not necessarily mean that the top-scoring sequence is a true relative of the query: it is quite possible that the database does not contain any relative at all. A number of strategies have been devised to address this question. A common basis is the statistical analysis of the score distribution that would be expected if the database contained only random sequences. For each score obtained in the actual sequence comparison an "expectation value" or "*E*-value" is then calculated, which corresponds to the probability that the given score is the result of a chance match alone. Low *E*-values are indicative of significant matches, a value of 0.01 would correspond to a 1% chance of being a mere coincidence and thus a 99% chance of being meaningful.

Heuristical or exact database search methods, combined with a rigorous statistical analysis of the scores obtained, are very useful tools for identifying relatives to given sequences, with the profile and HMM methods being the most sensitive ones. The two latter approaches have the additional advantage of allowing an "iterative refinement" process[11]. In the first step, a profile or Hidden Markov Model is calculated from a starting family of proteins. In the second step, the resulting profile or HMM is used for a database search. Database sequences that give significant scores and are not members of the initial family can, after carrying out some additional tests, be considered new members of the family. The expanded family now contains more sequence diversity and thus offers more possibilities to discrim-

inate the important regions from the less important ones. A new profile or HMM calculated from the expanded family can then be used for further rounds of database searches, until no new proteins with significant scores are found any more. This iterative process has been very successful in detecting even extremely distant sequence relationships with residue identities <15%.

5.3
Enzyme-specific Conservation Patterns

An iterative profile refinement search, as discussed in the previous section, frequently starts with a relatively small set of sequences with readily visible sequence homology. The multiple alignment will typically contain many conserved residues, some of them being conserved because of their crucial importance for the protein's structure or function, and others being conserved just for the reason that the genes encoding the proteins did not have enough time to diverge. Subsequent runs of the refinement process will pick up more distantly related proteins, decreasing the number of "chance conservations". When the iterative process has finished and the protein family under study contains a sufficient number of members and sequence diversity, most of the invariant residues will have a particular reason for being that well conserved.

The next paragraphs try to interpret frequently occurring classes of "conservation patterns", meaning the set of totally invariant or at least highly conserved positions in a family alignment. The conservation patterns of enzyme families are different from those of non-catalytic proteins and can be used for enzyme identification and classification.

5.3.1
General Conservation Patterns

When analyzing a large number of solved three-dimensional protein structures, it becomes evident that the amino acids buried in the internal regions of the structure are mainly non-polar and form the so called "hydrophobic core". By contrast, residues that are exposed on the surface and thus in contact with the solvent tend to be hydrophilic. Data available on structural flexibility also indicate major differences between the rigid core region and the flexible surface. If the conservation patterns of typical protein families are analyzed, a further trend becomes visible. Residues contributing to the hydrophobic core tend to be much better conserved than residues exposed on the outside. As a consequence, highly conserved residues are mostly hydrophobic. There are two other classes of residues that are frequently found to be invariant: glycine and proline. The reason for this preference is again based on the structure. A number of secondary structure elements, such as β-turns, require a very small residue like glycine. Proline too is required for particular structural elements since it introduces some rigidity to the backbone.

In general it can be stated that structural reasons contribute most to the

determination of the average protein family's conservation pattern. Hydrophobic residues tend to be highly conserved but not invariant, since they can frequently be replaced by related hydrophobic residues. Glycine, proline and, in the case of disulfide-bridges, also cysteine tend to be invariant or nearly invariant at structurally unique positions. The multiple alignment of a typical non-catalytic protein region is shown in Fig. 5-2 A.

5.3.2
Active Site Conservation Patterns

The structure of enzymes is governed by the same principles as that of every other protein. However, in addition to the structurally important residue conservation, enzyme families also have the tendency to conserve their active site residues highly. As will be discussed below, most enzyme families have retained a common reaction mechanism and thus a common set of catalytically important residues. As a consequence, active site positions are not only well conserved but mostly invariant. The set of residues found in catalytic centers of enzymes consist mainly of amino acids that can be protonated and/or deprotonated, or those able to form hydrogen bonds. The exact set of residues depends on the nature of the catalytic mechanism, but serine, cysteine, histidine and aspartate are particularly frequent. In addition, lysine, arginine, glutamate, threonine and tyrosine are occasionally found. In several enzyme classes, the high degree of conservation around the active center extends into a second layer, consisting of residues involved in orienting the catalytic side chains by forming a network of hydrogen bonds. As an example of a typical enzymatic conservation pattern, the multiple alignment of the duplicated but very compact catalytic region of phospholipase D type enzymes[29] is shown in Fig. 5-2 B.

5.3.3
Metal Binding Conservation Patterns

A number of proteins contain metal ions, which may serve either a structural or functional role, or even both[30]. In some proteins, the metal is bound by a particular cofactor, such as haem; other enzymes use the side chains of amino acids for coordinating the metal ion. While bound metals are not restricted to enzymes, a substantial proportion of hydrolases contain Zn^{2+} and other heavy metal ions, which typically contain one unoccupied coordination site that is used for binding and thus activating the substrate to be hydrolyzed. Similarly, a number of redox enzymes coordinate metal ions that are able to change their oxidation state, such as $Fe^{2+/3+}$ or $Cu^{+/2+}$. Prominent members among the proteins that bind metals for non-catalytic purposes are zinc-fingers, which frequently bind to DNA or to other proteins, and Ca^{2+}-binding EF-hand proteins, which serve mainly regulatory purposes.

Not all amino acid side chains make good ligands for metal ions. Acidic residues such as aspartate and glutamate are frequently found to coordinate small metal ions like Ca^{2+} or Mg^{2+}, while cysteine, histidine and aspartate are frequently involved in

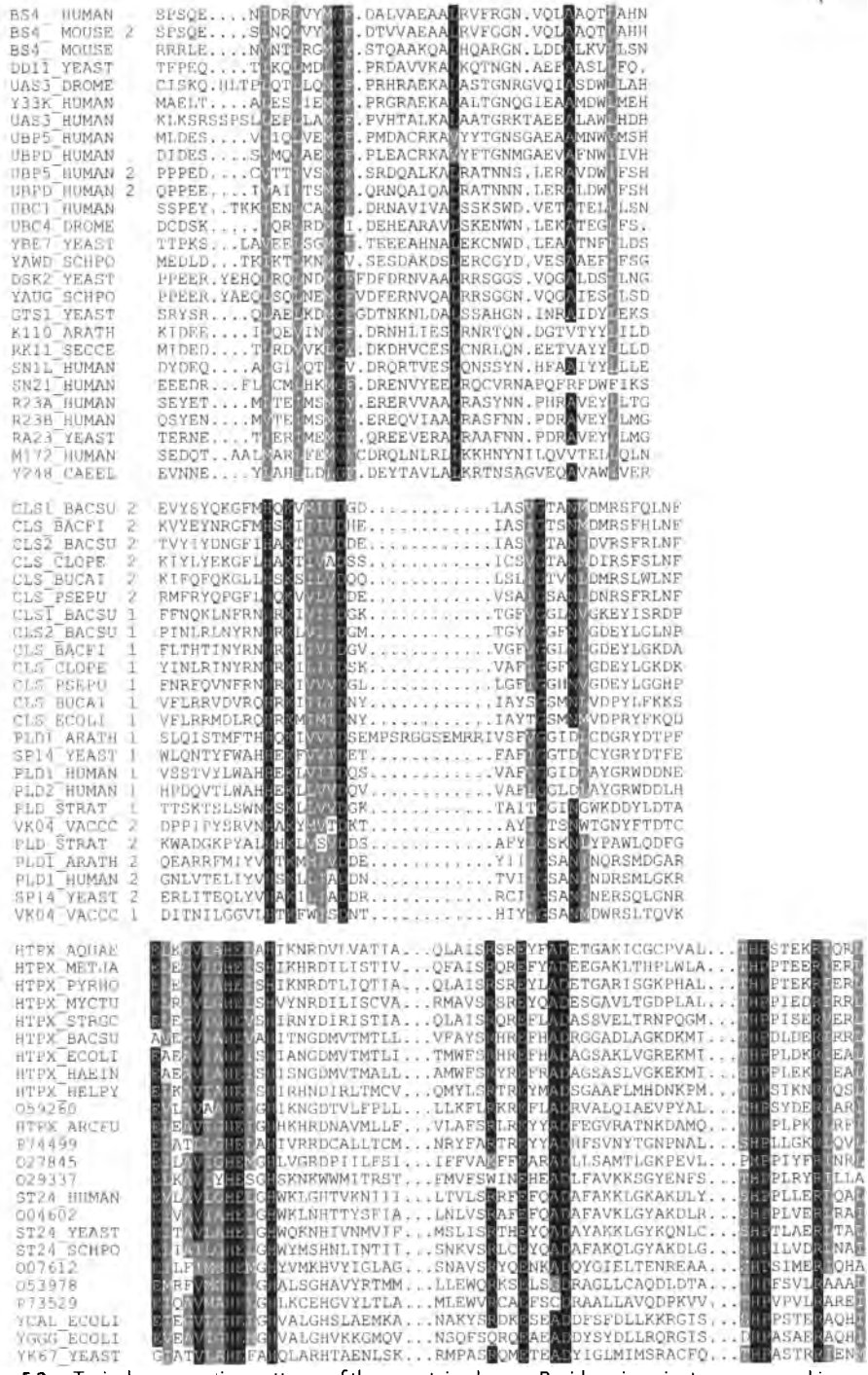

Figure 5-2. Typical conservation patterns of three protein classes. Residues invariant or conserved in more than 80% of the sequences are printed on a black or grey background, respectively. A: Mainly non-polar conservation in the UBA domain, a small protein domain that interacts preferentially with ubiquitin[78]. B: Invariant polar active site residues in the phospholipase D family[29]. C: Nearly invariant metal-binding residues in the HtpX/Ste24 family of Zn-containing metalloproteases.

coordinating Zn^{2+} or heavy metal ions. Just as with the amino acids participating in catalytic conversions, those coordinating metal ions fulfil a specialized role and tend to be invariant within protein families. If substitutions are observed, they normally stay within one class of coordinating residues, such as Cys↔His or Cys↔Asp. Since all side chain bound metal ions require multiple ligands, the corresponding protein families usually have a characteristic conservation pattern consisting of several invariant positions of the mentioned residue classes. A typical example is shown in Fig. 5-2C.

5.3.4
Making Use of Conservation Patterns

From what was said in the previous paragraphs, it appears that the specific conservation pattern of a protein family can be used to predict whether the proteins are enzymes, bind metal ions, or rather have a structural or regulatory role. If the proteins are known to be enzymes, the conservation pattern can be used to predict which residues are part of the active site, and possibly also which catalytic mechanism is being used. For example, it would be straightforward to submit a family of structurally uncharacterized proteases to that type of analysis in order to find out whether they are serine proteases, aspartate proteases, metalloproteases, or if they belong to a different class. Moreover, it is possible to compare the family's conservation pattern with those of other, better characterized enzyme families; this approach will be discussed in more detail in Sect. 5.6.

There are, however, a number of caveats that apply to the analysis of enzyme-specific conservation patterns. As mentioned previously, the method can be expected to work only in those cases where the sequence family contains enough divergent sequences to discriminate between the important and non-important positions. The large amount of available sequence data from all phyla, in combination with sensitive comparison methods like the iterative profile technique, make it possible to meet this requirement quite frequently. In addition, the analysis is complicated by the presence of catalytically inactive members of enzyme families. There is a rapidly increasing number of reports on those "outsider" proteins, which in the course of evolution have acquired fundamentally different non-catalytic roles. Examples include the transferrin receptor, which is a metal-free and inactive member of an ancient metalloprotease family[31], and the neuroligins, which are inactive members of the choline esterase family[32]. Those proteins have no selection pressure to preserve the non-functional active site residues and, as a consequence, they are typically replaced by various structurally compatible amino acids. The presence of inactive members in a family alignment means that one can no longer expect a total invariance of the active site residues. Since the non-catalytic proteins usually replace not only one active site residue but rather all of them, there is the chance to identify inactive members or even inactive subfamilies by the concerted loss of conservation in the presumed catalytic positions.

Finally, there is a small number of cases, where members of an enzyme family have, in the course of evolution, assumed a different catalytic role, using a different

set of active site residues. An example of this situation is the enoyl-CoA isomerase/hydratase family (or crotonase family). The "inner family" comprises various enoyl-CoA hydratases, isomerases, epimerases and 4-chlorobenzoyl-CoA dehalogenases[33]. While these reactions are catalytically distinct, they all share the feature of using CoA-activated substrates and all of them utilize the same set of residues for catalyzing the first common step of the reaction[34, 35]. However, sensitive sequence comparisons demonstrate a more distant but nevertheless highly significant relationship to the ClpP enzymes, a class of bacterial proteases. This latter family does not use CoA activated substrates, catalyzes a totally different reaction and uses a distinct set of active site residues grafted onto a very similar structural core[36, 37]. In terms of conservation pattern analysis, this case can be treated similarly to the previous one, i.e. a coordinated loss or change in residue conservation has to be accounted for.

It has to be said that all of the mentioned complications should be considered exceptions rather than the rule. Overall, an analysis of conservation patterns has been and will continue to be a valuable tool in the identification and classification of new enzyme families.

5.4 Modular Enzymes

A survey of known three-dimensional structure of proteins shows that a sizeable portion of them contain several apparently independent folding units, usually referred to as "domains".

5.4.1 The Domain Concept in Structure and Sequence

A protein domain, in the structural sense, is a part of the whole protein that folds independently from the rest of the structure and has a hydrophobic core of its own. Residues lying within the domain are mainly in contact with other residues of the same domain; there are only few interactions between residues within and outside of the domain. In evolutionary terms, genes encoding multi-domain proteins can be explained as fusion products of simpler genes. Nature's main advantage of using a multi-domain organization of proteins is the possibility of having different functions assigned to different domains of a proteins, which can act more or less independently of each other. Functional domains that have proven useful can then, by an evolutionary process involving exon shuffling or gene fission/fusion events, be re-used in other proteins where they fulfil a similar function[38, 39]. Apparently, this modular approach to protein structure has been very successful: there are several functional domains that can, with only minor modifications, be found in more than 100 different proteins of one organism.

While the original definition of a protein domain is based on the structure, it is also possible to detect "re-usable modules" in protein sequences. Local regions of

sequence similarity, which are typically found in several proteins per organism, are called "homology domains" and usually correspond roughly to structural domains[40]. The self-sufficiency of protein domains makes it possible to insert them into almost any sequence context, thus giving rise to the sharp drop of sequence similarity at the domain boundaries. When comparing two sequences, the presence of a well-conserved homology domain, embedded into a totally unrelated context, makes it necessary to use "local" alignment methods as opposed to "global" ones. Local alignment algorithms do not require the total sequences to match with each other but rather score the best matching region within the sequences. All sequence comparison methods mentioned in Sect. 5.2 support a local alignment mode.

5.4.2
A Classification of Modular Enzymes

A modular architecture is no particular hallmark of enzymes, the highest degree of modularity is typically observed in structural proteins of the extracellular matrix and in proteins involved in intracellular signal transduction. Nevertheless, there are a number of modular enzymes including some which are of interest for biocatalytic applications.

A recent review of modular enzymes[41] mentions three different but not mutually exclusive types of modularity: i) the separation of substrate recognition and catalytic activity on different domains, ii) modularity in multi-substrate enzymes, which use different domains for binding the two or more substrates that are to react with each other, and iii) modular enzyme systems that catalyze several consecutive reactions of a metabolic pathway. When considering the biocatalytic usefulness of modular enzymes, the first type appears less relevant, since the typical substrate molecules are too small to allow a true spatial separation of recognition and reaction. In physiological situations, however, this type of modularity is highly important wherever the recognition and conversion of macromolecular substrates is concerned. The second type, i.e. modularity in multi-substrate enzymes, might offer some possibilities to change the components involved. If, in a family of transferases catalyzing different reactions, donor and acceptor moieties are recognized by different domains, it could be attempted to swap domains between family members and thus change the specificity of the enzyme, possibly even to one not observed in nature. Examples of this situation are the NDP-glycosyltransferases and the bacterial polyketide synthases.

The third type of modularity, the multi-catalytic enzymes using substrate channelling, are of particular interest for synthetic applications. Prominent members are the fatty acid synthases, the polyketide synthases and the non-ribosomal peptide synthases[42–44]. In these large proteins, a number of catalytic domains is combined with accessory domains and allows the catalysis of an entire pathway by a single polypeptide chain. Multi-catalytic enzymes frequently use a "swinging arm", which is covalently attached to the intermediary product of one reaction step, and is subsequently able to present this molecule to the next catalytic domain for further

processing[45]. As an example, a typical non-ribosomal peptide synthase contains one or more domains forming the "swinging arm", several domains for activating specific amino acids, several domains for catalyzing the transfer of the activated residues onto the growing chain, and one domain each for loading and unloading the swinging arm. Additional domains that catalyze further enzymatic steps such as redox reactions or cyclizations are also found. These enzymes, as well as the bacterial polyketide synthases, are promising tools for the biosynthesis of antibiotics and other related natural products. Part of the promise stems from the specificity of the activation reaction and from the fact that the sequence of the reaction process is encoded by the domain arrangement. It has been shown that artificially swapped domains can lead to active enzymes that now synthesize a different product[46, 47]. So far, not all domains occurring in those enzymes are fully understood by function, and not all attempted domain swappings have lead to viable enzymes. Nevertheless, multifunctional re-programable enzymes and other engineered hybrid-enzymes will undoubtedly have an interesting future in biocatalytic applications[48].

5.4.3
Inhibitory Domains

Besides the three types of modularity mentioned, there is a fourth type that is very useful in a physiological setting but tends to be undesired *ex vivo*. In a living cell, an uncontrolled enzymatic activity at the wrong place or the wrong time can be deleterious. To avoid is type of complication, many enzymes have acquired inhibitory domains, which are encoded by the same polypeptide as the enzyme itself. Whenever in the biological system the enzymatic activity is needed, the inhibitory region is cleaved off or is neutralized by other methods, e.g. by binding to an activator protein. Biocatalytic applications typically require permanently active enzymes. Thus, it is desirable to recognize inhibitory domains and remove them before using the enzyme. As mentioned above, bioinformatic methods such as sequence comparisons can help to find those domains and to determine, with some confidence, the likely domain boundaries.

5.5
Enzyme Databases and Other Information Sources

Now that the principles of enzymatic architecture and the corresponding analysis strategies have been highlighted and briefly discussed, an overview of the existing enzyme classes and their properties is needed. Given the more than 4000 different enzyme types, any attempt at only listing them would be far beyond the scope of this chapter. Fortunately, there are a number of specialized databases available, which aim to treat various aspects of enzyme structure and function comprehensively. All of these databases are accessible via the Internet, and a list of the relevant URL addresses is given in Table 5-1.

5.5.1
E.C. Nomenclature and ENZYME Database

The widely accepted basis of all enzyme classifications are the recommendations of the Enzyme Committee (E.C.) of the International Union of Biochemistry and Molecular Biology (IUBMB)[49]. Within this system, enzymatic activities are classified by a four-level hierarchy and each entry is described by a set of four numbers. The first number describes the top level and can be either "1" for oxidoreductases, "2" for transferases, "3" for hydrolases, "4" for lyases, "5" for isomerases or "6" for ligases. The meaning of the three lower hierarchy levels depends on the top level group. As an example, glycogen synthase is classified as 2.4.1.11; here, the "2" stands for transferases, the "4" for glycosyl-transferases, the "1" for hexosyl-transferases and the "11" for the particular subfamily.

The ENZYME database[50], maintained by the Swiss Institute for Bioinformatics (SIB), provides a comprehensive list of all IUBMB classifications, together with associated information such as systematic and alternative enzyme names, cofactor requirements, and pointers to the corresponding entry in the SWISS-PROT database of protein sequences[51]. In addition, there is a concise free-text description of the reaction catalyzed, together with a description of preferential substrates and products. Currently, the ENZYME database holds entries for approximately 3700 enzymes.

5.5.2
BRENDA

A much more ambitious database that builds on the IUBMB classification is BRENDA, maintained by the Institute of Biochemistry at the University of Cologne. In addition to the data provided by the ENZYME database, the BRENDA curators have extracted a large body of information from the enzyme literature and incorporated it into the database. The database format strives to be readable by both humans and machines. The categories of data stored in BRENDA comprise the EC-number, systematic and recommended names, synonyms, CAS-registry numbers, the reaction catalyzed, a list of known substrates and products, the natural substrates, specific activities, K_M values, pH and temperature optima, cofactor and ion requirements, inhibitors, sources, localization, purification schemes, molecular weight, subunit structure, posttranslational modifications, enzyme stability, database links, and last but not least an extensive bibliography. Currently, BRENDA holds entries for approximately 3500 different enzymes.

From the wealth of information presented, it is clear that BRENDA is a very important resource for enzymes in organic synthesis.

5.5.3
KEGG and LIGAND database

The Kyoto Encyclopedia of Genes and Genomes (KEGG) is an effort to reconstruct biological pathways from the gene repertoire found in the genome sequencing projects[52]. The LIGAND database is an associated database of enzymes and their reactions, which is also hosted by the University of Kyoto[53]. LIGAND consists of three different but interconnected segments. The COMPOUND section holds 5600 entries of various compound classes with relevance to enzymatic reactions (substrates, products, inhibitors etc.). The ENZYME section contains 3400 entries corresponding to the enzymes themselves. Finally, the REACTION section contains approximately 5200 reactions. In combination with the KEGG/PATHWAY database, the data stored in LIGAND are not only presented in static form but can also be used to calculate biological pathways between a given substrate and product.

5.5.4
UM-BBD

The University of Minnesota Biocatalysis/Biodegradation Database (UM-BBD) is a data repository providing curated information on microbial catabolic enzymes and their organization into metabolic pathways[54]. At present, the UM-BBD stores information on approximately 100 pathways with 700 reactions, 600 compounds and 400 enzymes. The database does not try to cover every known enzyme but rather focuses on those used for the biodegradation of xenobiotics. UM-BBD is linked to the ENZYME, BRENDA and KEGG/LIGAND databases mentioned above.

5.5.5
Structural Databases

Although not being in the focus of this chapter, structural databases are a most useful resource for the scientist interested in enzymes and reaction mechanisms. The Protein Data Bank (PDB) is the main repository for all three-dimensional structures of macromolecules including enzymes[55]. Nowadays, most journals accepting manuscripts that describe new structures require a simultaneous deposition of the structural coordinates with the PDB database. In addition to the structure of single protein molecules, the PDB also contains several entries of multi-protein complexes, or proteins bound to small-molecule compounds.

Of the 14 500 entries currently in PDB, there are roughly 7200 enzyme structures. The Enzymes Structures Database, maintained by University College, University of London, focuses on this portion of PDB and offers links between the E.C. nomenclature of the IUBMB and the corresponding PDB entries.

5.5.6
Metalloprotein Databases

As mentioned in Sect. 5.3.3, a number of enzymes contain metal ions that participate in the catalytic reaction. Two specialized databases store information on metal ions and other bioinorganic motifs in enzymes. PROMISE (prosthetic centers and metal ions in protein active sites) is maintained by the University of Leeds and focuses on six major groups of metal containing proteins: diiron-carboxylate proteins, haem proteins, iron-sulfur proteins, molybdopterin proteins, mononuclear iron proteins, and chlorophyll containing proteins [56].

The Metalloprotein Database and Browser (MDB) is maintained by the Scripps Research Institute and aims to collect quantitative information on all metal containing sites available from structures in the PDB database [57]. The data stored comprises both structural and functional information on the metals bound and the ligands involved. The associated database server allows specific queries for particular site geometries and functions.

5.5.7
Databases for Selected Enzyme Classes

In addition to the above mentioned databases that try to cover the entire world of enzymes, there are a number of more topical databases focusing on particular enzyme families. The MEROPS database, maintained at the Babraham Institute in Cambridge, provides a catalog and a structure-based classification scheme for all proteolytic enzymes [58]. In addition to the classification, the database also provides a digest of published information on the peptidases as well as cladograms and multiple sequence alignments of the peptidase families.

The ESTHER database, maintained at the INRA-ENSAM in Montpellier, follows a similar concept but focuses on the α/ß fold family of esterases/lipases [59].

5.6
Protein Domain and Motif Databases

As has been described in Sect. 5.3, the conservation patterns of enzymes are often indicative of the particular family they belong to and can be used for their classification. However, the iterative searches and multiple alignment methods used for their establishment require a certain bioinformatic infrastructure as well as some experience with these issues. If the goal of the analysis is not the detection of novel enzyme families, but rather the classification of a novel sequence into one of the already existing enzyme families, there are a number of protein domain and motif databases that will be useful in this respect [60, 61]. These databases do not store the sequences themselves but rather work with "descriptors" of protein families and protein domains. These descriptors can consist of the Profiles or Hidden Markov Models mentioned above, but other types are also being used. With a particular

search engine, typically provided with the databases, it is possible to scan one or more unknown protein sequences against large libraries of pre-defined family or domain descriptors. These search engines are publicly accessible via the Internet; the relevant addresses are listed in Table 5–1. Currently, none of the available databases has a particular focus on enzymes. Nevertheless, a substantial proportion of the databases discussed below consist of enzyme families or of catalytic or regulatory enzyme domains.

5.6.1
PROSITE

The PROSITE database, maintained by the Swiss Institute of Bioinformatics (SIB), was the first database that tried to catalog functional motifs and domains of proteins[62]. Nowadays, PROSITE consists of two major parts storing different types of descriptors: the "pattern" library and the "profile" library[63].

The pattern entries of the PROSITE database are based on a regular expression syntax, which emphasises only the most highly conserved residues in a protein family, corresponding approximately to what is termed a "conservation pattern" in Sect. 5.3. In contrast to the other databases mentioned below, PROSITE patterns do not attempt to describe a complete domain or even protein, but rather try to identify the functionally most important residue combinations, which in enzymes typically correspond to the active site. As an example of the PROSITE syntax, "K-x(1,2)-[DE]" would mean a lysine residue, followed by one or two arbitrary residues, followed by a residue that is either aspartate or glutamate. When a sequence is compared with a library of such patterns, any pattern is found to be either present or absent, no intermediate scores are assigned. Currently, the PROSITE pattern libraries contains approximately 1400 entries.

A consequence of the rigid syntax of PROSITE patterns is the restriction that they work well only with those protein families that really contain invariant or at least highly conserved positions. When dealing with catalytic sites of enzymes, this requirement is usually met. However, a large number of protein families and domains are too divergent to be appropriately described in the framework of a regular expression syntax. To circumvent that problem, the PROSITE curators introduced another section of the database using generalized profiles as descriptors[64]. As mentioned above, profiles are based on preferences for particular amino acids rather than on strict requirements. Thus, profiles are suited better for highly divergent protein families and domains, but require a different search engine. An important factor contributing to the usefulness of PROSITE is the extensive documentation of the entries, discussing e.g. the active site residues or the phylogenetic scope of a motif, and also providing links to other databases and to the literature.

5.6.2
PFAM

PFAM is a database of Hidden Markov Models of protein families and domains, maintained at the Sanger Centre in Cambridge[65]. The concept of PFAM is comparable to that of the PROSITE profile section. Similar to the profiles, the HMMs in PFAM have been derived by the iterative refinement procedure mentioned in Sect. 5.2.4. Unlike the PROSITE profiles, which all have been created manually by the curators, the HMMs in PFAM are generated semi-automatically, which accounts for a slightly lower sensitivity. However, this lack is more than compensated for by the facilitated update procedure, allowing the database to grow much faster than PROSITE and to have a shorter generation cycle. Currently, PFAM holds 2727 entries.

5.6.3
Other Related Databases

A number of other protein motif databases should not be left unmentioned. The SMART database is conceptually very similar to PFAM, but the collection of Hidden Markov Models focuses on proteins involved in intracellular signal transduction[66]. The PRINTS and BLOCKS databases are similar to PROSITE and PFAM in that they do not have a thematic focus[67, 68]. However, unlike the databases mentioned above, their motif descriptors recognize short non-gapped regions of the proteins. Several other protein motif- and domain-databases and their application in the classification of proteins have been reviewed recently[60, 61]. The INTERPRO consortium, consisting of the curators of various protein domain databases, is currently developing a non-redundant combination database, offering a common search interface[69].

A fundamentally different approach is used by PROCAT, which does not describe motifs in linear sequence but rather structural motifs, i. e. combinations of residues that occur in a similar position in the 3D-structure of protein family members[70, 71].

5.7
Enzyme Genomics

The last few years have seen a rapid increase in the number of completely sequenced genomes; an even greater number of whole genome sequences is near completion. Currently, 49 genome sequences have been published in the scientific literature, and both their DNA sequence and the protein sequence of the predicted gene products are in publicly accessible databases. A taxonomic breakdown of the completed genome sequences shows that five of them belong to eukaryotes, nine to archaea and 35 to eubacteria. So far, the choice of the organism for the genome projects has been based mainly on the general scientific interest or on their biomedical importance. A number of organisms selected for their technological interest are being sequenced as

well. However, the driving force behind those genome projects are mainly commercial entities, raising the question of when and to what extent the sequences will be made known to the public. Databases like GOLD provide information on both finished genomes projects and those underway[72].

What is the relevance of genome sequences to the search for biocatalytically applicable enzymes? At least two different avenues, in this context called "ortholog search" and "paralog search" have the potential to yield results that are immediately useful.

5.7.1
Ortholog Search

A number of enzymes from microbes or other organism are considered useful but not totally satisfactory for synthetic applications. Frequently encountered problems include lack of stability, too low catalytic rate, too broad or too narrow specificity, and poor availability of the natural or recombinant enzyme. In these cases, it might be favorable to replace the enzyme by an ortholog from another organism, i.e. by an enzyme that fulfils exactly the same role in another species and is related to the original enzyme in the same way as the corresponding organisms. One possible rationale for this approach is that not all orthologs in a family have exactly the same properties, thus there is a certain likelihood of finding a "better" enzyme in another species by chance alone. A more targeted approach for finding "better" orthologs can also be envisaged: if e.g. the goal is to increase the thermal stability of an enzyme, the orthologs from thermophilic organisms are prime candidates for the desired improvement[73]. In general, different life environments and slight differences in metabolic pathways give rise to certain variations in an enzyme's properties, which can be exploited in the search for optimized enzymes.

An obvious prerequisite for this type of optimization is being able to find orthologs to given enzymes. A second requirement is that the (sequence derived) ortholog pair has not evolved so far that they catalyze different reactions. When dealing with completely sequenced genomes, the search for othologs is frequently straightforward. A number of complications have been described[74], the most frequent being that the gene of interest has been duplicated in one of the lineages. In the cases of absent one-to-one ortholog relationships, it is more appropriate to speak of orthologous groups of genes rather than of ortholog genes. The COG database, maintained at the NCBI, has defined orthology clusters for the publicly available genome sequences and is updated whenever new genome sequences become available[75].

5.7.2
Paralog Search

A more demanding problem in the bioinformatic mining of genome sequences is the search for truly novel enzymes. A possible starting point would be the knowledge that a particular organism possesses an enzyme with the desired specificity, while the corresponding protein sequence is elusive. In order to address this type of

question, several approaches are conceivable. One of them is based on the analysis of conservation patterns and phylogenetic relationships in large, non-orthologous enzyme families, and will be discussed in the following paragraph. Other methods, which are not based on sequence homology at all, are highlighted in Sect. 5.7.3.

When analyzing the sequence/function relationship in multiple enzyme families such as those collected in PROSITE and PFAM, a number of general rules emerge. It has been mentioned previously that the catalytic mechanism and the active site residues of an enzyme are better conserved than the overall sequences. In most cases, the same is true for the region of the substrate that is modified in the course of the reaction, in particular for the type of bond that is being broken or formed. There is a general trend that related enzymes catalyze identical or closely related reaction types but not necessarily with related substrates. While there are numerous examples of this trend, there are only a few counter examples. Almost all members of the α/β fold lipase family catalyze the hydrolysis of carboxyl-esters (or the reverse reaction), no matter whether the substrates are lipids or polar compounds. Similarly, there are several families of phosphoesterases, that act on substrates as diverse as phospholipids, phosphoproteins and nucleic acids, but invariantly cleave a phosphoester bond. Multiple families of acyltransferases exist, which have as a unifying criterion the nature of the acceptor atom (O, N, S) rather than a common recognition feature in the substrates. Among the very few counter examples are the enzymes of fatty acid ß-oxidation. As mentioned in Sect. 5.3.4, the enoyl-CoA hydratase and isomerase catalyze different reactions but use a very similar substrate. This particular example can, however, be explained by a common activation step in both reactions.

This knowledge can be exploited to search complete genome sequences for proteins that encode enzymes of a given specificity. In the first step, the enzymatic reaction under question has to be analyzed for the nature of the atoms involved and the bonds to be formed or broken. In the second step, the available knowledge base of enzymes and enzymatic reactions has to be screened for any relatives. Useful in this respect are the databases of Sect. 5.5, like ENZYME and BRENDA, which already have classified enzymatic reactions by the necessary criteria. In addition, the protein motif databases of Sect. 5.6 might already have assembled a family of enzymes that catalyze the desired reaction type. If, in this process, a known enzyme is found to catalyze a reaction with a similar mechanism to the desired one, this enzyme sequence can be used for a paralog search in the third step. The expression "paralog" applies to evolutionarily related proteins, either within one species or between species, that are not "orthologs", i.e. that do not directly correspond to each other. Paralog pairs are expected to catalyze similar reactions instead of identical ones. Finally, in the fourth and last step, the found paralogs can be assumed to be candidates for the missing enzyme and their activity can be verified experimentally.

Since paralogs are typically more distantly related than orthologs, their detection frequently requires sensitive protein comparison methods such as profiles or HMMs. Even the detection of orthologs can, under some circumstances, require sophisticated database searching methods, e.g. if the corresponding organisms belong to very distant phyla.

5.7.3
Non-homology Based methods

The methods described in the previous section are all based on homology, i.e. a recognizable sequence relationship caused by a common evolutionary descent. An additional approach to identify candidate genes for a given enzymatic function does not rely on homology, but rather on a peculiarity of bacterial genome organization. Bacteria tend to have proteins belonging to one metabolic pathway clustered in a contiguous stretch of the genome, all present in the same transcriptional orientation. The reason for this clustering is an economy of transcriptional regulation. In most cases, the components of a pathway have to be expressed in a coordinated fashion. This regulation is greatly facilitated by the "operon" arrangement, where multiple bacterial genes are under the control of a single promoter.

Again, this knowledge can be exploited when searching for an unknown enzyme with a known involvement in a particular pathway. The first step is the identification of other proteins likely to work in the same pathway as the desired enzyme. In the second step, the genome of the target organism is searched for genes encoding those upstream or downstream components. In the third step, other genes belonging to the same operon(s) are identified and treated as enzyme candidates unless a different function can be assigned to them. This "operon-approach" to enzyme identification is particularly useful in situations were the gene in question cannot be identified by sequence similarity, e.g. in cases of "non-orthologous gene displacement". This expression describes a phenomenon that is occasionally observed in bacterial genome comparisons[74, 76]. Here, two organisms use similar pathways, where most but not all of the genes involved have a clear one-to-one relationship. The remaining genes might catalyze exactly equivalent reactions, but are not related at all because the two organisms have recruited members coming from different protein families for an identical task.

Not all bacterial genes in general, and enzymes in particular, are organized in operons. A prerequisite for the method described above is a reliable detection of operons and the participating genes. Again, evolutionary considerations can help: if related genes, preferably orthologs, occur in a conserved order in several bacterial genomes, this is a clear indication of an operon organization and thus most likely also of a functional coupling. Computer databases of genome organization, such as the STRING system maintained at the EMBL, are useful tools for detecting those relationships[77].

5.8
Outlook

In the recent years, at least two developments have made major contributions to the field of enzyme bioinformatics. One of them, the advent of whole genome sequencing, is widely recognized for its impact on virtually every field of biochemistry and molecular biology. By contrast, the development of sensitive sequence comparison

methods has remained largely unnoticed, although it has made possible a new level of understanding genomic data. The most useful databases of protein families and domains, together with their associated search systems, would not have been possible without profile and Hidden Markov Model methods.

These two achievements work synergistically. On one hand, the sequence comparison and classification approaches are required for an efficient functional assignment of genome sequences and also for inter-genome comparisons. On the other hand, the iterative refinement process relies on sequence diversity within protein families and can make use of the genomic data, even in its raw and functionally uncharacterized state. At present, only a fraction of known enzymatic domains and protein families is covered by databases such as PROSITE and PFAM. Within the next years, this fraction will increase, since more genome data will probably uncover a large amount of new enzymes, accompanied by only a minor increase in the number of truly new enzyme families. Eventually, we will see a nearly complete coverage of enzyme families, which will greatly facilitate the identification and classification of any new enzyme sequence that becomes available.

A field that will most certainly gain influence in the next years is that of "structural genomics". Several attempts have been initiated to elucidate the three-dimensional structure of an organisms entire protein complement, or at least a substantial fraction of it. While the results coming from these projects will open a straightforward path to fold recognition, the value for enzyme bioinformatics might not be as high as it might seem. The most useful structural information on enzymatic mechanisms comes from structures where the enzyme is analyzed while binding to a substrate analog or to an inhibitor. These studies, however, require *a priori* knowledge on the enzymatic properties and the nature of the substrate, which is not available in "blind" high-throughput studies.

Another area of intensive research in the field of applied genomics is the gene expression analysis by DNA microarrays and similar methods. As of now, most applications of these techniques are either based on their scientific merits or on medial/pharmaceutical/toxicological applications. It is probably only a matter of time until these methods find their way into research on biocatalysis. Possible applications include the analysis of coordinated regulation of enzymes not linked in operons, or the identification of new enzymes on the basis of their expression pattern .

As in all other areas of bioinformatics, databases will play an increasingly important role in managing and integrating the data coming from various sources. A database system meant to be useful for the exploitation of enzymes for synthetic applications would have to encompass information on organisms, their genome sequences and their metabolic pathways, with a special emphasis on the enzymes involved, their reaction types and the nature of the substrates and products. Databases such as KEGG and others have already started to address these questions. However, none of the currently available genome- and pathway-databases are focused on biocatalysis, a fact that will certainly change within the next couple of years.

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6
Immobilization of Enzymes

James Lalonde and Alexey Margolin

6.1
Introduction

Readers of this text are well aware of the promise of enzyme catalysis for the elegant synthesis of complex molecules. However, the practical application of enzymes as catalysts for organic synthesis is often limited by the inherent differences between the way that molecules are synthesized by biological systems and the way they are prepared on the laboratory bench. Nature has designed enzymes to catalyze reactions under physiological conditions, most often in aqueous media at ambient temperature and pressure and at neutral pH with dilute concentrations of reactants. Preparative chemical syntheses, in contrast, usually require high concentrations of reactants and the use of organic solvents to dissolve organic substrates and to shift reaction equilibria. Isolation of organic products from water can be complicated by the presence of an amphiphilic protein. While biological systems destroy and regenerate enzymes as they are needed, catalysts used in chemical manufacture must often be recovered and reused many times for economic viability. Immobilization of an enzyme is the most commonly used strategy to impart the desirable features of conventional heterogeneous catalysts onto biological catalysts.

By definition, enzyme immobilization is the conversion of an enzyme to a form with artificially restricted mobility and retention of catalytic function[1]. This restricted mobility allows for containment and recovery of the enzyme and is often achieved by either conversion to an insoluble form (for example by linking to insoluble particles) or by containment within a semi-permeable barrier (for example entrapment within an ultrafiltration membrane). In the course of this immobilization, enzymes can acquire four advantageous properties:

- Immobilized enzymes can be used repeatedly or continuously in a variety of reactors.
- They can be easily separated from soluble reaction products and unreacted substrate, thus simplifying work-up and preventing protein contamination of the final product.

- The catalytic properties, pH-activity profile and enzyme stability can be enhanced in the immobilized form.
- The control of microbial contamination in solid immobilized preparations is often simpler than for soluble protein.

There are, however, a number of practical limitations on the utility of immobilized enzymes. First, the yield of protein binding is rarely quantitative. Second, in many cases, the cost of the carrier can be quite significant and may even exceed the cost of the enzyme itself. Third, the activity of the resulting immobilized enzyme is usually reduced because of chemical modification of the protein, steric hindrance and mass transfer limitations. Finally, the proportion of active enzyme to the carrier material in immobilized enzyme preparations rarely exceeds 5–10% w/w, and thus dramatically reduces catalytic activity per weight of solid.

Despite the limitations, the great success of enzyme immobilization in diagnostics, pharmaceutical, food and chemical industries is undeniable[2, 3]. The decision whether one should use a soluble enzyme preparation or an immobilized enzyme does not have a universal solution and can be decided only on a case by case basis. Ordinarily, if the cost of an enzyme represents a significant portion of the overall cost or if isolation of the final product is complicated by the presence of the soluble protein, the cost of immobilization can be offset by the gains in productivity and improved product quality. The intent of this section is to describe, in general terms with illustrative examples, the features and considerations of these broad classes of enzyme immobilization as they impact their application to biocatalysis. Detailed experimental protocols are available in the original literature and exemplary protocols for these methods are offered in many excellent reviews and texts[4].

6.2 Methods of Immobilization

Thousands of publications and patents detail the immobilization of specific enzymes using an impressive array of strategies. The majority of these immobilization techniques can be divided into four broadly defined groups:

- non-covalent adsorption of an enzyme onto a solid support;
- covalent attachment of an enzyme to a solid support;
- entrapment of an enzyme in a polymeric gel, membrane or capsule;
- cross-linking of an enzyme with a polyfunctional agent.

The first three classes involve the use of a solid matrix to support or entrap the enzyme and to confer the desirable mechanical properties of the solid carrier (Fig. 6-1 A-D). The last method entails covalent linking of the enzyme to itself with no additional support (Fig. 6-1 E). Each of the covalent methods requires one or more covalent bonds between reactive groups on the enzyme surface with complementary groups on the carrier, either directly or through the action of a multivalent cross-linking reagent. Covalent attachment methods result in direct chemical modification

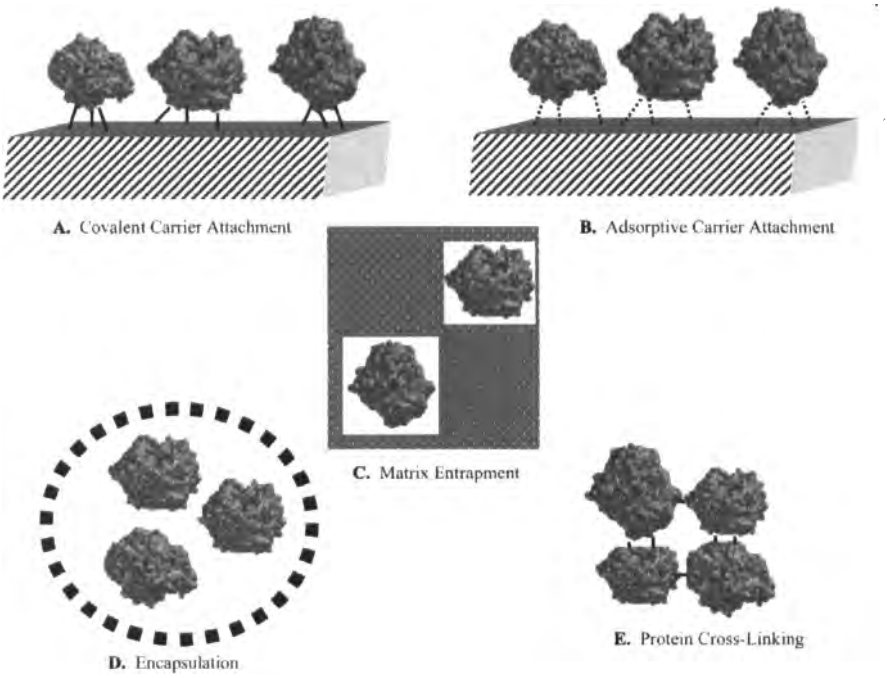

Figure 6-1. Classification of Immobilization Methods.

of the protein molecule. Non-covalent methods are based on formation of an enzyme/carrier complex through simple physical confinement or by electrostatic attraction, hydrogen bonds, van der Waals interactions, and so-called hydrophobic interactions. Matrix entrapment (Fig. 6-1 C) and encapsulation (Fig. 6-1 D) are both considered to be methods of entrapment in this chapter. A summary of the advantages and disadvantages of each of these four classes of immobilization is given at the end of this section in Table 6-1.

6.2.1
Non-Covalent Adsorption

Adsorption of an enzyme to a solid carrier is characterized by the interaction of a protein with a solid surface through reversible, non-covalent binding. The interaction forces in adsorption processes range from relatively strong ionic and hydrogen bonding to weaker van der Waals forces and "hydrophobic" interactions of the protein with the support. Electrostatic forces of ionic and hydrogen bonding are much stronger than purely hydrophobic ones, and so can afford a tightly bound protein, even in purely aqueous media. Immobilization by adsorption has the advantage of simplicity, is often inexpensive, and does not usually result in disruption of the catalytic protein structure. No chemical modification of the protein or

support occurs; however, binding to the carrier is reversible and leaching of the protein can be a problem. Moreover, in cases where the binding forces are weak, there is little stabilization of the enzyme tertiary structure relative to the solution form of the enzyme. Interaction of the reaction substrate or products with the support can cause desorption of the adsorbed enzyme. This reversibility of binding can in some situations be advantageous; if the protein catalyst has become inactivated from extended use, the resin can be regenerated by a change in pH or solvent to desorb the deactivated enzyme from the carrier and then fresh biocatalyst added under binding conditions. While more exotic or expensive proteins often warrant the use of covalent binding methods, adsorption is most often used in large scale industrial processes because of the low cost and simplicity.

Electrostatic binding of enzymes to polyionic carriers is operationally simple, once appropriate binding conditions are identified. The carrier is first equilibrated using aqueous media at the appropriate pH, solvent composition and ionic strength. An aqueous solution of the enzyme is then treated with the adsorptive solid under conditions of protein concentration, pH, ionic strength, and temperature that have been determined experimentally to give efficient protein binding. If the binding protocol is relatively selective, the immobilization can also effect purification of the enzyme from cell debris and fermentation by-products. The immobilized biocatalyst can be recovered by filtration and then washed. Air drying or washing with a water-miscible organic solvent can be used to give a dried biocatalyst solid for use in non-aqueous media. A disadvantage of this method is that the support materials used in ionic adsorption are polyfunctional and charged and thus can dramatically change the microenvironment of the protein. Steric hindrance to diffusion of substrate and product can also be a problem, due to the short protein-support distance in tight ionic bonding.

Ionic adsorption of proteins is one of the oldest methods of protein immobilization and has been used widely in industry. Chibata and co-workers developed one of the earliest industrial biocatalytic processes using an amino acylase adsorbed on diethylaminoethyl (DEAE) carbohydrate resin for the kinetic resolution of amino acids[5]. Macroporous synthetic ion exchange resins, based on those originally developed for chromatography and water treatment, are among the most frequently used carrier materials. The protein is bound through association with the side chains of amino acids such as aspartate and glutamate (carboxylate) and lysine (ammonium) through oppositely charged groups on the carrier. The tightness of binding is dependent on the proximity and charge of the binding residues on the protein surface and the carrier. Protein binding can be quite tight if factors which affect ionization such as pH, counter-ion identity, hydrophobicity and ionic strength are optimal.

A demonstration of the principles of ionic adsorption is found in the use of glucose isomerase bound to DEAE-cellulose[6] for the conversion of glucose syrup to high fructose corn syrup. Remarkably little enzyme desorption of glucose isomerase is observed over many months, despite the use of elevated temperatures and high flow rates through columns of the resin-bound enzyme. During the lifetime of the catalyst, 1 g of catalyst converts 15 000 g (dry substance) of high fructose corn syrup.

However, once inordinate activity has been lost, the protein can be easily removed by a simple shift in pH and then the resin regenerated *in situ*.

Lipases from *Candida antarctica*, *Humicola lanuginosa*, and *Mucor meihei*, useful for enantioselective ester hydrolysis or tranesterification, have also been immobilized by ionic attachment to synthetic resins[7]. For the interesterification of fats and oils, macroporous (>100 Å pore diameter) methacrylate resin cross-linked with divinylbenzene gives virtually quantitative binding of the protein. The air-dried resin can be used to catalyze interesterification of oils in the absence of solvent[8]. The preparation of *Candida antarctica* B lipase has been widely used for the resolution of carboxylic acids and alcohols[9].

Ionic attachment to nonionic surfaces can be effected through the intermediacy of a polyvalent metal cation[10]. Chelation of a transition metal by both the carrier surface and the enzyme results in binding to the surface. Inorganic oxides (such as silica) or polyhydroxylated biopolymers (such as polysaccharides) are used as solid supports in combination with polyvalent transition metals capable of binding multiple ligands such as Ti(V). This type of chelation binding is also used extensively in the isolation of genetically engineered proteins by incorporation of a polyhistidine tag sequence. The poly-His sequence chelates tightly to Cu(II) or Ni(II), providing a selective means for selective recovery of the protein[11].

Affinity binding is an important sub-group of ionic protein adsorption methods. Specific electrostatic and hydrophobic interactions between the target enzyme and an immobilized ligand allow for extremely tight, selective binding of the protein of interest. The ligand may be a small molecule or a large protein such as an antibody; however, the loading capacity tends to decrease with the effective molecular weight of the ligand. One of the most frequently used affinity binding systems is the combination of biotin with the protein avidin. Avidin is a tetrameric protein which binds four biotin ligands by specific ion-pair interactions with a dissociation constant of about 10^{-15} M. In a typical embodiment, biotin derivatives that are linked to a reactive functional group are covalently attached to both the solid surface and to the protein. The biotinylated solid is first treated with avidin, and this is followed by treatment with the biotinylated enzyme[12]. The expense and necessity for extensive manipulations make this method of affinity binding practical only for aqueous systems and those using very highly valued enzymes.

Immobilized enzyme preparations that are bonded only through strictly nonionic, physical adsorption are rare; however, in non-aqueous systems physical adsorption can be a very effective approach. In purely hydrophobic binding, the protein molecule is not solvated by the bulk reaction solvent sufficiently to overcome the weak interaction forces with the solid surface, and so the protein does not desorb from the carrier. In many of these non-aqueous systems, there is thought to be activation of the protein by the support providing a more hydrophobic environment which facilitates wetting and interaction with the non-polar substrate and by distribution of the enzyme over a larger surface area. Alternatively, activation of the lipase enzyme by interaction of hydrophobic regions on the protein with the hydrophobic surfaces have been postulated. Dispersion of lipases over a high-surface hydrophobic polymeric carrier such as polypropylene or nylon has been

shown to activate this enzyme in organic solvent media relative to particles of the untreated protein prepared by lyophilization. Hydrophobic binding of lipases is sufficiently strong to allow their use in purely aqueous media, presumably because of the affinity of this protein for water/oil interfaces. Patel has reported that a lipase immobilized on polypropylene could be reused for ten cycles without loss of activity in the kinetic resolution of a key intermediate for semi-synthetic Taxol[13].

Adsorption on polar, nonionic carriers represents the middle ground in non-covalent attachment, where a combination of hydrogen bonding and dipole interactions helps to bind the protein to the support. The immobilized lipase used in the upgrading of low value fats and oils by interesterification is a successful example of this mode of non-covalent adsorption. In one example, an aerosol of an aqueous solution of the lipase is sprayed onto finely divided silica and then the particles are agglomerated to give the particulate biocatalyst. The simplicity and effectiveness of this adsorption process afford a dried immobilized biocatalyst with sufficient productivity to be used on a manufacturing scale at relatively high temperatures[14].

6.2.2
Covalent Attachment

The immobilization of enzymes by covalent attachment to a solid carrier involves formation of a covalent bond between amino acid side chain residues of the protein with reactive groups on the support surface. Covalent attachment is often the method of choice where the protein value is high, minimal protein leaching from the support is required or rational control of the biocatalyst properties is desired. Because of the stronger carrier-protein linkage, the resulting heterogeneous biocatalyst can be much more stable than those prepared by adsorption or entrapment. The most common protein functional groups involved in covalent bonding are nucleophilic amino (lysine, hisitidine and arginine), thiol (cysteine) and hydroxyl groups (serine, threonine and tyrosine) and electrophilic carboxylate groups (aspartic acid and glutamic acid). The reactivity of these functional groups can be modulated through chemical modification, but this can be detrimental to activity and the extra degree of complexity is not often warranted.

Rational control of properties of the immobilized biocatalyst is possible with covalent binding; by choice of the reactive functional group on the support and control of its distribution, the practitioner can control the nature and degree of protein modification and the microenvironment of the immobilisate. Binding with minimal loss of catalytic activity is thought best to occur with residues on the surface of the protein and should involve groups that are remote from the active site of the enzyme to avoid deactivation. In the preponderance of cases, primary amino groups on the protein surface are coupled with electrophilic groups on the support material. Surface-exposed lysine and arginine residues are allowed to react with electrophiles via alkylation, conjugate addition, imine formation or acylation. Alkylation and conjugate addition proceed with retention of the net protein charge. Less frequently, carboxylate residues on the enzyme are activated for reaction with nucleophilic functional groups on the carrier. Figure 6-2 depicts some of the more commonly

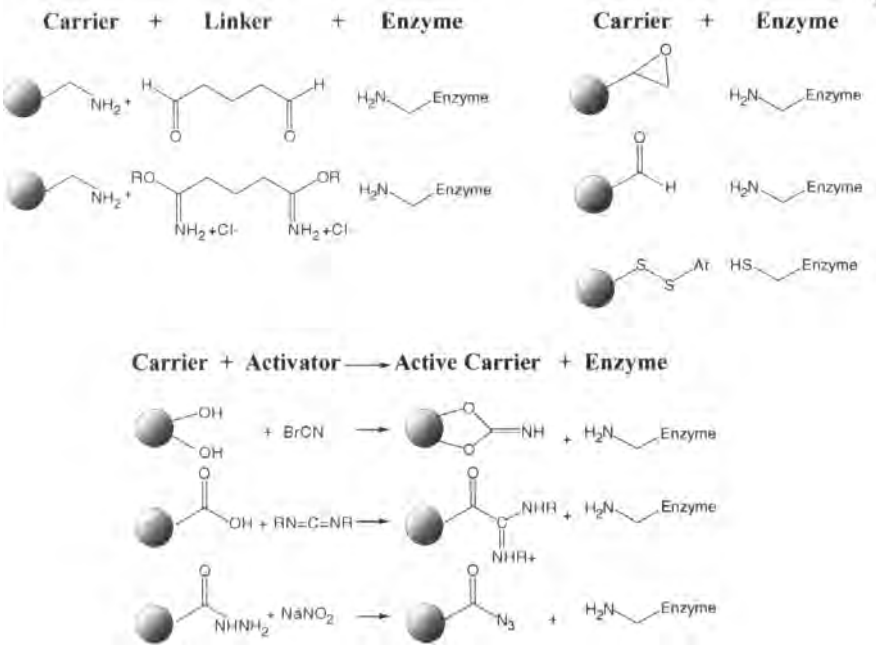

Figure 6-2. Examples of Common Carrier Activation Methods.

used combinations of reactive protein groups and activated supports [15]. The choice of reactive group is important; highly reactive groups may result in non-specific over-modification, and chemical functionalization that adds or removes charge from the protein can alter the activity and stability. There are many examples of more limited and specific attachment methods using reagents selective for less common amino acids. No one support and linker is ideal and the large number of supports and binding method leads to an enormous number of possible permutations.

The preparation of covalently bound immobilized enzymes involves treatment of a solution of the protein with the reactive support. Judicious choice of conditions including enzyme concentration, pH, and ionic strength can be used to increase the yield of bound activity. The loading capacity of the carrier can be estimated from the manufacturer's specifications or by titration with reagents specific for the reactive functional group. A competitive inhibitor or high concentration of substrate may be used during attachment to protect the active site and to maintain the active conformation of the enzyme. After incubation, the resin is washed repeatedly to remove unbound protein, and then the free reactive sites are quenched by treatment with an appropriate nucleophilic or electrophilic reagent (for example, glycine or acetic anhydride).

Either the solid support or the enzyme may be activated, but to limit disruption of the enzyme tertiary structure the functional groups of the support material are most often activated. The activation may occur prior to the coupling reaction (pre-activated supports), or a bi-functional linking reagent may be used to form the bond between

Table 6-1. A Comparison of immobilization methods.

Immobilization Method	Advantages	Disadvantages
Adsorption	• Simple • No chemical modification of enzyme • Reversible • Often inexpensive	• Weak binding, leaching of enzyme • Little or no stabilization • Non-specific binding • May limit mass transfer
Covalent	• Tight binding • Wide variety of supports and linkers available • Rational control of enzyme loading, distribution and microenvironment	• Chemical modification of enzyme • Often expensive • Activity diluted by carrier • May limit mass transfer
Entrapment and Encapsulation	• No chemical modification of enzyme • Can be simple • Efficient for whole cells	• Little or no stabilization • Environmental changes can disrupt network and cause leakage • Often limits mass transfer
Cross-linking	• High volumetric activity • Compatible with elevated temperature and organic solvents • No carrier required • Tight binding • Efficient for whole cells	• Chemical modification of enzyme • Little control of particle properties (especially for precipitate and whole cell) • Requires crystallization of enzyme (for CLEC®) • May limit mass-transfer

protein and support. A comparison of the various immobilization methods is given in Table 6-1.

6.2.2.1
Carriers for Enzyme Immobilization

The physical and chemical properties of protein molecules are often not compatible with the conditions used in most chemical syntheses, and so fixation to a solid carrier is one effective strategy to alter the properties of the biocatalyst. By binding of the protein to a proportionately large amount of a solid carrier, the bulk properties of the resultant solid biocatalyst are more derived from the carrier than from the protein. Enzymes are subject to denaturation conditions found in typical chemical processing such as high concentrations of organic reagents and high shear forces. Moreover, proteins are water soluble and amphipathic thus causing emulsions on extraction and being difficult to recover and reuse. The carrier-fixed biocatalyst is often more resistant to deactivation by organic reactants or shear and can be recovered by simple filtration. In many cases the recovered biocatalyst maintains catalytic function and may be reused many times.

An enormous number of carriers are available for the immobilization of enzymes

Table 6-2. Carrier types.

Organic – synthetic polymer	Organic – biopolymer	Inorganic
Polyamides	**Polysaccharide**	**Minerals**
• Nylon	Cellulose	Sand
Polyalkylene	Starch	Pumice
• Polystyrene	Agarose	Metal oxides
• Polyacrylates	Dextran	Diatomaceous earth
• Polyacrylamide	Chitin	Clays
• Polyethylene	Polyalginate	
• Polypropylene	Carrageenan	
• Polyvinyl alcohol	**Proteinaceous**	**Synthetic**
• Polyvinylacetate	Gelatin	Glass, controlled pore glass
• Polyvinylchloride	Collagen	Zeolites
• Polyethylene glycol	Silk	Silica
Polyester	Albumin	Sol-gel
• Polycarbonate	Bone	Alumina
Polyurethane		Metal Oxides
Polysiloxane		Metals
Phenol-formaldehyde		

and a wide range of methods have been used for fixation of protein to these carriers. For rapid preparation of laboratory samples, commercially available pre-activated macroporous resins are available. Considerations of the desired properties of the immobilized biocatalyst such as ease of use, mechanical strength, activity density, stability, intended application, cost, and availability help to determine which carriers and methods of attachment are appropriate. In most industrial applications, cost of the support and efficiency of immobilization are paramount, while in biomedical applications binding efficiency and ability to sterilize can be most important. Classification of materials used in solid carriers is given in Table 6-2.

When the mass of carrier material is large relative to that of the enzyme, the physical and chemical properties of the carrier (Table 6-5) will, in large part, determine properties of the resultant immobilized enzyme. Often, the carrier will impart mechanical strength to the enzyme, allowing repetitive recovery by simple filtration of the solid particles and reuse of the enzyme. The degree of porosity and pore volume will determine the resistance to diffusion and molecular size selectivity of the biocatalyst. When used in non-aqueous media, dispersion of the enzyme over a large surface area can greatly increase its activity. Table 6-3 summarizes many of the key properties and considerations for enzyme carrier materials.

6.2.3
Entrapment and Encapsulation

Entrapment can be defined as any system in which an enzyme or whole cell is physically restricted within a confined space or network. This class of immobilization is often extended to include systems where a combination of physical entrap-

Table 6-3. Summary of properties and considerations for enzyme carriers.

Property	Examples or typical range	Characteristics and considerations
Binding mode	Covalent, ionic or physical adsorption. Pre-activated or activated in situ.	Binding strength, enzyme stabilization, ease of use, protein charge
Shape	Bead, flat sheet or hollow fiber membrane, amorphous aggregate Crystal	Ease of filtration, Control of diffusion path length and flow properties Simple preparation
Surface area	>50 m^2/g	Binding capacity, volumetric activity
Porosity, pore size	>1 ml/g, 2–50 nm	Resistance to diffusion, molecular weight sieving, flow properties, enzyme retention
Particle size and distribution	1 μm to 1 mm	Ease of filtration, sedimentation velocity
Density		Resistance to diffusion, sedimentation velocity
Safety		Sterility, toxicity, regulatory approval for food and drug use, consumer and worker exposure
Mechanical strength		Resistance to shear, compression, tearing of membranes or particle fracture
Compressibility	Rigid particle to soft gel	Ease of filtration and handling, column packing
Solvent compatibility		Swelling, dissolution of carrier, enzyme desorption, controlled dissolution
Hydrophobicity and charge	Hydrophobic to polyionic	Alteration of substrate selectivity, shift of pH/rate optimum, enzyme stabilization, binding force and capacity
Reactive site distribution	Evenly distributed or only on surface	Surface vs. bulk attachment, multipoint attachment, stabilization
Loading capacity	0.1 % to 10 % w/w	Enzyme/carrier ratio, volumetric activity
Cost	"Free" to 1 000s of USD/g	Economics, availability for scale-up, catalyst productivity and lifetime

ment and covalent binding is used. Entrapment immobilization includes enzymes contained within such diverse systems as polymeric matrices, hollow-fiber ultrafiltration membranes, liposomes, cross-linked arrays, or cross-linked whole cells. Depending on the density of the entrapment matrix, the environment of the protein can be similar to that of the protein in the bulk reaction media, and disruption of catalytic activity is relatively minor. The pore structure of the matrix used for

entrapment is such that small molecules (substrates and products) are able to diffuse in and out of the matrix, while the macromolecular enzyme is maintained within the network. Mass transfer limitations are almost always an issue with entrapped enzymes and whole cells, since precise control of pore size is usually not possible. Often a certain fraction of the enzyme is able to diffuse from the network, and swelling of these molecular networks by a change in reaction conditions can accelerate this leakage of protein.

Entrapment of enzymes or whole cells in a cross-linked polymeric network can be achieved by a number of methods. The most common methods of gelation of a polymer or pre-polymer include:

- Cross-linking of a pre-formed polymer or formation of a polymer network in the presence of the biocatalyst;
- Solvent-, temperature-, or pH-induced precipitation;
- Addition of multivalent cations to a polyacid.

Polyacrylate and polyacrylamide gels have been found to have favorable properties for the entrapment of enzymes and whole cell biocatalysts[16]. These gels are sufficiently hydrophilic to provide an environment similar to that of the bulk aqueous solution. Acrylamide or methacrylate monomers, for example, can be polymerized in the presence of enzymes and polyfunctional cross-linkers to form a gel-entrapped biocatalyst preparation. The stiffness of the gel and pore size can be controlled by the amount and type of cross-linker used. Higher degrees of cross-linking and short spacer groups give a stiffer gel, while longer spacer groups give larger pores. The average molecular weight of the gel can be influenced by the amount of free radical initiator used, the reaction time and the temperature of polymerization. The particle size can be controlled by mechanically cutting the particles to the desired size or by performing the polymerization under emulsion polymerization conditions.

Rhodococcus sp. microorganisms which express high levels of nitrile hydratase have been entrapped in polyacrylamide and polyacrylate resins for the conversion of acrylonitrile to acrylamide[17]. Limitations common in cell entrapment such as resin swelling, deactivation during the entrapment, mass transfer limitations of substrate and product were addressed by the control of mixing rate during polymerization, gel density, particle size and resin hydrophobicity. Activation of carboxylate residues in the polymer matrix by conversion to the hydrazide improved retention of the enzyme, presumably through the covalent attachment of lysine side chains on the enzyme surface via amide linkages[18].

Gelation of polyanionic or polycationic polymers by the addition of multi-valent counter-ions is a simple and common method of entrapment of enzymes and whole cells. In one common embodiment, whole cells or enzymes are entrapped by the drop-wise addition of an aqueous solution of sodium alginate and the biocatalyst to a concentrated solution of a Ca^{2+} salt. The cation acts as a cross-linking agent towards the alginate biopolymer and the droplets precipitate as beads with the biocatalysts entrapped within the network. Although the beads are relatively soft and unstable, this method has been one of the preferred methods for entrapment of whole cells. A

second commonly used example of this technique, gel formation using κ-carrageenan in the presence of high concentrations of potassium salts, has been used for the immobilization of asparatase producing cells for the production of L-aspartic acid [19]. Similarly, carrageenan entrapment of yeast cells has been used on an industrial scale for the production of malic acid by the action of fumarase on fumaric acid [20]. Leakage of enzyme is often a problem in these systems, especially on exposure to ion-complexing agents such as phosphate buffer. The mechanical properties and enzyme retention can be improved by treatment with glutaraldehyde or other covalent cross-linking reagents.

The term encapsulation has been used to distinguish entrapment preparations in which the biocatalyst environment is comparable to that of the bulk phase and where there is no covalent attachment of the protein to the containment medium (Fig. 6-1 D) [21]. Enzymes or whole cells may be encapsulated within the interior of a microscopic semi-permeable membranes (microencapsulation) or within the interior of macroscopic hollow-fiber membranes. Liposome encapsulation, a common microscopic encapsulation technique, involves the containment of an enzyme within the interior of a spherical surfactant bilayer, usually based on a phospholipid such as lecithin. The dimensions and shape of the liposome are variable and may consist of multiple amphiphile layers. Processes in which microscopic compartmentalization (cf. living cells) such as multienzyme systems, charge transfer systems, or processes that require a gradient in concentration have employed liposome encapsulation. This method of immobilization is also commonly used for the delivery of therapeutic proteins.

Enzyme-membrane reactors represent an interesting subset of macroscopic enzyme entrapment [22]. A semi-permeable ultrafiltration membrane with a sufficiently low molecular weight cut-off restricts passage of the enzyme to the bulk substrate and product phase. In these reactors, the soluble enzyme can be used in a continuous fashion as the product isolation is isolated in a separate vessel from the enzyme. Progress in this application has been facilitated by the availability of solvent-resistant membranes with tighter pore size distributions. The membrane can be used to simply separate the enzyme and bulk substrate and product phases or to separate the aqueous enzyme phase from an organic phase containing substrate and product. The resolution of L-methionine by the enantioselective hydrolysis of *N*-acetyl-L-methionine has been performed on the scale of hundreds of tonnes/year in a continuous process using soluble amino acylase in a membrane reactor [23]. An extension of this strategy to cofactor restriction was effected by coupling the cofactor nicotinamide adenine dinucleotide (NAD^+) with polyethylene glycol to increase its molecular weight. Co-entrapment of the pegylated cofactor with the soluble enzymes leucine dehydrogenase and formate dehydrogenase in the asymmetric reductive amination of trimethylpyruvate to L-*tert*-leucine [24] allows thousands of turnovers of the expensive cofactor. In the synthesis of the key chiral intermediate for Diltiazem, a lipase entrapped an asymmetric hollow fiber membrane performs the kinetic destruction of the undesired enantiomer. The membrane serves to maintain an aqueous environment for the enzyme and an interface between the buffer phase and that of an organic phase which contains the substrate phenylglycidate ester [25].

6.2.4
Cross-Linking

Immobilization by chemical cross-linking without the addition of an inert carrier or matrix can provide the means to stabilize and reuse a biocatalyst without dilution of volumetric activity. A major deficiency in all of the aforementioned immobilization methods is that a substantial amount of a catalytically inert carrier or matrix is used to bind or contain the biocatalyst. In many cases, the amount of carrier is two orders of magnitude higher than the protein catalyst. Unfortunately, direct cross-linking of the enzyme, followed by precipitation of an amorphous solid often results in low activity and poor mechanically properties and so this method is not often used. Recently, however, cross-linked enzyme crystals have been reported to give many of the desirable properties of immobilized enzymes without the need for a support material (Sect. 6.4.1).

Chemical cross-linking of an enzyme within its host cell is a simple and economical method to produce an entrapped or encapsulated biocatalyst, eliminating the need for isolation or purification of the enzyme. Whole cells may be lysed or left intact and then chemically cross-linked by the addition of polyfunctional reagents such as glutaraldehyde or toluene diisocyanate. The mechanical properties of such preparations are poor, but can be improved by the addition of support matrices such as gelatin or synthetic organic polymers (which, technically, are considered to be entrapment methods). Cross-linking of whole cells is an effective entrapment method for relatively stable enzymes that do not require additional stabilization of the support matrix. One of the largest industrial biocatalytic processes, that to produce high fructose corn syrup, can employ the biocatalyst as a cross-linked whole cell preparation. A patent assigned to Novo[26] describes the immobilization of glucose isomerase via entrapment of the lysed cells of the host organism within a cross-linked network of glutaraldehyde and, optionally, an alkyl diamine.

6.3
Properties of Immobilized Biocatalysts

As with most heterogeneous catalysts, it is often difficult to characterize immobilized enzymes at a molecular level. Most immobilized preparations are often complex mixtures with a distribution of chemically modified protein species. The gross catalytic properties observed are a composite of those of a range of differentially modified individual proteins, often irregularly distributed within the sample. Mass transfer limitations and microenvironment effects further complicate characterization.

6.3.1
Mass Transfer Effects

The catalytic behavior of enzymes in immobilized form may dramatically differ from that of soluble homogeneous enzymes. In particular, mass transport effects (the transport of a substrate to the catalyst and diffusion of reaction products away from the catalyst matrix) may result in the reduction of the overall activity. Mass transport effects are usually divided into two categories – external and internal. External effects stem from the fact that substrates must be transported from the bulk solution to the surface of an immobilized enzyme. Internal diffusional limitations occur when a substrate penetrates inside the immobilized enzyme particle, such as porous carriers, polymeric microspheres, membranes, etc. The classical treatment of mass transfer in heterogeneous catalysis has been successfully applied to immobilized enzymes [27]. There are several simple experimental criteria or tests that allow one to determine whether a reaction is limited by external diffusion. For example, if a reaction is completely limited by external diffusion, the rate of the process should not depend on pH or enzyme concentration. At the same time the rate of reaction will depend on the stirring in the batch reactor or on the flow rate of a substrate in the column reactor.

The extent of internal mass-transfer is usually expressed by the efficiency coefficient N,

$$N = V_{imm}/V_{sol}$$

where V_{imm} and V_{sol} are the rates of the reaction catalyzed by an immobilized and soluble enzyme, respectively. In order to find out whether a reaction is limited by diffusion one can calculate n as a function of the Thiel modulus (Fr)

$$Fr = R \times (k_{cat}[E]/D_{eff}K_m)^{1/2}$$

Where R is the carrier radius, D_{eff} the effective diffusion coefficient of the substrate, E is the enzyme concentration in the carrier, and k_{cat} and K_m are the kinetic parameters of an enzyme. From a practical standpoint it is important to remember that there are no diffusional limitations as long as substrate concentration S exceeds K_m. This condition normally exists at the beginning of many processes catalyzed by immobilized enzymes. At the end of the process, when a substrate is depleted and effective K_m may increase because of the product inhibition, the whole reaction may be limited by diffusion.

6.3.2
Partition

The other important phenomenon that, in addition to the mass transfer, occurs when enzymes become heterogeneous catalysts, is the partitioning of substrates, products, inhibitors, metal and hydrogen ions between a bulk solution and a carrier. An elegant and simple theory describing the effect of microenvironment inside the particles of immobilized enzymes on their kinetics, has been developed by the group

of Katchalsky[28]. In particular, the theory explains why one often observes shifts in pH profiles of activity with immobilized enzymes; it is due to the redistribution of hydrogen ions between a bulk solution and a carrier. As a practical consequence, one should use a negatively charged carrier if a shift of pH profile to a more alkaline pH is desired and a positively charged carrier if the opposite shift to an acidic pH region is necessary. However these electrostatic effects exist in solutions with low ionic strength and almost disappear when salt concentration increases. In general, the partitioning of substrates and products between a solution and a carrier may occur whenever the character of a carrier (charge, hydrophobicity, etc.) differs from that of a bulk solution. As a result, the binding constants for substrates (K_m) and for products (K_i) with immobilized enzymes may vary dramatically from those observed for free enzymes.

6.3.3
Stability

One of the chief benefits of enzyme immobilization is the ability to use them repeatedly in chemical reactors on a large scale. Usually this cannot be achieved without a significant increase of enzyme stability. It is clear that attachment of an enzyme to a solid surface greatly limits deactivation by intermolecular protein-protein processes such as aggregation or proteolysis. In some cases, this is the only stabilization provided by immobilization. In other cases, immobilization leads to stabilization of the three-dimensional catalytic structure against intramolecular protein denaturation under conditions such as elevated temperature, extremes of pH, organic solvents and oxidants. Protein unfolding can be prevented by multipoint attachment of a protein to a support; however, it is not clear whether this increase in rigidity is generally beneficial to catalytic function. As an approximation, the optimal immobilization is given by the maximum functionalization which results in minimal activity loss. Over modification of the enzyme often results in loss of activity and stability. In some specific cases, covalent multipoint attachment of a protein to a solid carrier clearly enhances the resistance to chemical and thermal denaturation[29]. An increase in the number of both polar (electrostatic) and hydrophobic interactions among the protein molecules when a protein goes from a free to immobilized environment may also significantly enhance stability of proteins against heat and other denaturants[30] by preventing unfolding, aggregation or dissociation of the proteins[31]. Moreover, observed stabilization effects correlate with the number of contacts involved[32]. The intermolecular cross-linking of proteins by glutaraldehyde and other cross-linking agents[33] may, in turn, lead to additional thermostabilization of proteins by preventing their unfolding.

6.3.4
Activity of Immobilized Enzymes

On the surface the activity assay of immobilized enzymes is quite simple and is not very dissimilar from measuring the activity of soluble enzymes. In both cases the

Table 6-4. Parameters for characterization of immobilized biocatalysts.

General description	Preparation	Physical and chemical characterization	Kinetics
• Reaction scheme • Enzyme and source • Carrier type • Method of immobilization	• Method • Detailed reaction conditions • Dry weight yield • Activity left in supernatant • Enzyme leakage, conditions	• Particle shape, diameter, swelling • Compression in columns or abrasion in stirred vessels or sedimentation and abrasion in fluidized bed	• Initial rate versus concentration for free and immobilized enzyme • pH and buffer effects • Diffusional limitations • Effect of particle size and enzyme loading • Degree of conversion versus residence time • Storage stability • Operational stability

activity is measured in μmol of substrate per minute per gram of a catalyst under defined conditions (concentrations, pH, temperature, etc.). Yet, the heterogeneous nature of immobilized enzymes poses additional challenges. First, special care should be taken in choosing a representative sample of an immobilized enzyme. Second, the activity of immobilized enzymes is much more sensitive to external parameters, such as stirring, and may be limited by diffusion (see above). Third, the determination of true catalytic parameters is more difficult, since the amount of the active enzyme attached to the carrier is not easy to measure. One has to realize that the k_{cat}, K_m and K_i measured for immobilized enzymes often represent the effective parameters. This is further complicated by surface activation effects in lipases[34].

The complexity of the physical and catalytic properties of immobilized biocatalysts and the difficulty in comparison of effectiveness based on literature descriptions has led to the publication of guidelines for the characterization of immobilized biocatalysts[35]. The authors suggest that description of parameters listed in Table 6-4 should be the minimum required for characterization of an immobilized preparation.

6.4 New Developments and Outlook

Opportunity for innovation and creativity still exists in the field of biocatalyst immobilization. Despite the tremendous volume of biocatalyst immobilization literature, there is no one technology that is universally applicable and no one technique that can be applied using a generic procedure. The limitations of individual immobilization techniques have been pointed out in each section. Operationally simple adsorption methods often are limited by the lack of stabilization and by protein leaching, especially under aqueous conditions. Restriction of diffusion can be severe for entrapped proteins and cells. Covalent methods often result in protein inactivation and a much higher carrier cost. The combined effects of

inefficiency in protein binding, carrier expense, protein inactivation on binding, restricted substrate diffusion, enzyme leaching, and enzyme denaturation during use can result in a tremendous overall activity loss and increase in cost when compared to the native biocatalyst. For example, with most current carrier-fixation technologies, Rasor[36] estimates that a 10- to 25-fold overall increase in cost can be expected in converting an enzyme to its immobilized form. Recent work in the field of biocatalyst immobilization has focused on the development of more efficient systems that employ relatively inexpensive support materials (see for example[14]), and in some cases, no support at all (Sect. 6.4.1).

6.4.1
Cross-linked Enzyme Crystals (CLEC®)

Early work in protein X-ray crystallographic structure determination demonstrated that protein crystals could be stabilized by cross-linking with glutaraldehyde[37]. More recently, cross-linked enzyme crystals (CLEC®) have been shown to be highly active and stable heterogeneous biocatalyst preparations[38]. In this method, a polyfunctional cross-linking agent is allowed to diffuse into a protein crystal such that the protein is cross-linked throughout the entire particle. In this case the enzyme is not linked to a carrier, but to adjacent enzyme molecules within the crystal. The protein itself is thus both catalyst and carrier. Electrostatic and hydrophobic contacts within the crystalline lattice, combined with added covalent cross-linkers, help maintain protein activity and stability in aqueous and organic media. It has been proposed that a higher degree of chemical functionalization is possible than with attachment to a two-dimensional surface because the added protein-protein contacts within the crystal particle stabilize the tertiary structure.

Immobilization by cross-linking of enzyme crystals appears to be a generic method; however, unique protocols must be developed for each individual protein. Preparation of a CLEC form of many types of proteins and classes of enzymes have been reported including hydrolases, oxidoreductases, carbon-carbon lyases and isomerases. Crystallization of the protein is a highly effective purification step, so

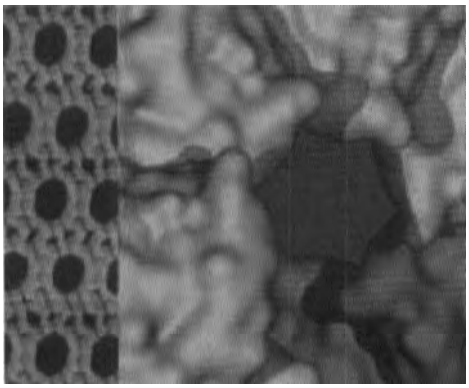

Figure 6-3. Graphic Comparison of 6 Å Zeolite B Channel (A) and 21 Å Thermolysin Crystal Pore (B).

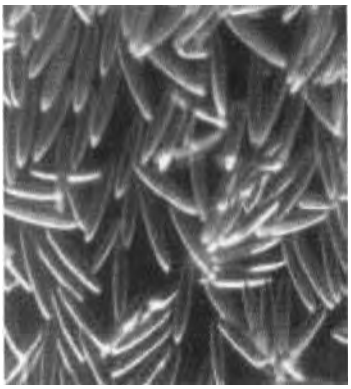

Figure 6-4. Cross-Linked Enzyme Crystals® of Thermolysin, Average Length 40 μm.

that undesired side activities can also be eliminated[39]. However, conversion of a soluble protein to a cross-linked enzyme crystal form requires development of procedures of protein crystallization that are specific for each protein, so that no generic protocol can be applied. The high degree of porosity (average of 50%) and large pore diameter (20–100 Å) of most protein crystals allow relatively unrestricted diffusion of small organic molecules (<2000 Da) within the crystal. This unrestricted diffusion, combined with the absence of an inert carrier, results in maximal volumetric activity for CLEC immobilized enzymes. As with all immobilized biocatalyst particles, mass transfer limitations can become appreciable as the rate of catalysis increases, as molecular radius increases and when larger crystal particles are used. The rate of enzyme catalysis in organic media for hydrolases in CLEC form, long considered to be two or three orders of magnitude lower than catalysis in water, have been shown in some instances to be equal to or greater than that of the corresponding hydrolytic reaction[40]. Cross-linked enzyme crystals of lipases[39], proteases[41] and penicillin acylase[42] have been made on the multi-kilogram scale and used in industrial processes to produce tonnes of drug intermediate per day[43]. Cross-linked enzyme crystals, in contrast to untreated protein crystals or cross-linked amorphous precipitate, are mechanically tough. In high shear mixing or repetitive filtration cycles of the CLEC form of penicillin acylase, there was no observable particle attrition. Kasche and Tischer have compared the relative benefits of CLEC immobilization and traditional carrier fixation. The authors conclude that CLEC enzymes follow the same kinetic laws as traditionally immobilized enzymes and that the chief benefits are the lack of inert carrier, operational stability and high volumetric activity[44].

An alternative approach to CLEC technology, the cross-linking of protein in solution followed by precipitation or freeze drying has been used for some time to avoid the use of inert carrier (see Sect. 6.2.4). Recently it has been reported by Sheldon that a cross-linked enzyme aggregate (CLEA) formed by first precipitation of penicillin acylase using salt or alcohols followed by chemical cross-linking gives an insoluble enzyme preparation with excellent activity and stability in water and organic solvents[45].

6.4.2
Sol-Gel

Reetz[46] and others[47] have found that entrapment of lipases within a hydrophobic silica sol-gel can result in a biocatalyst whose activity in organic media is enhanced in comparison to the corresponding lipase powder under the same conditions. A silica matrix is generated in the presence of an aqueous solution of the lipase by treating hydrophobic alkyl alkoxysilanes with a catalytic amount of sodium fluoride. The gel is allowed to set, then dried and crushed to the desired average particle size. Optimization of lipase activity can be achieved through variation of the hydrophobicity of the gel by choice of the alkyl group of the silane monomer, the use of hydrophilic polymeric additives (polyvinyl alcohol or inert proteins), control of water activity and by the water/silane monomer ratio. Activation of up to two orders of magnitude in the rate of fatty acid esterification relative to the suspended protein powder have been attributed to the combined effects of dispersion over a large surface area and the interaction of hydrophobic regions of the polysiloxane gel with hydrophobic domains of the lipase. The volumetric activity of these systems can be quite low: protein loadings of 0.1–1 % relative to the carrier are necessary for high activation factors. Moreover, the lipase is for the most part passively entrapped within the gel, so its use would be limited to non-aqueous systems. Organopolysiloxanes carrying pendant groups for covalent binding can be employed for immobilization catalysts intended for aqueous systems[48].

More recently, Ballesteros has extended the use of siloxane gel supports by developing support systems employing glyceryl poly(alkylsiloxanes)[49] and poly-(hydroxymethylsiloxanes) for the gel entrapment of lipases. The authors point out that the higher water solubility of these polymers allow for better control of the protein-polymer ratio. Mechanical properties and protein retention of the preparation were improved by entrapping the poly(hydroxylmethylsiloxane) lipase adsorbates within a solid cross-linked silicone rubber matrix. The versatile chemistry of silicone polymers allows the tailoring of the hydrophobicity and rigidity of the support matrix. Lipase loadings of 1–5 % are described for the poly(hydroxymethyl)siloxane/silicone polymer composites. Entrapment efficiencies are apparently sufficient to retard protein leaching; however, most aqueous reaction systems reported employed an organic co-solvent which limits enzyme dissolution. These lipase/polymer composites could be used for the kinetic resolution of racemic carboxylic acids and alcohols via ester hydrolysis or synthesis with negligible loss of activity over 10 reuse cycles.

6.4.3
Controlled Solubility "Smart Polymers"

Enzymes are normally used as water-soluble homogeneous catalysts or immobilized onto water-insoluble solid supports. The many advantages of immobilized systems have been outlined in this chapter. Yet insoluble immobilized enzyme preparations can also have serious drawbacks. First, efficiency with macromolecular or insoluble

substrates such as proteins or polysaccharides is often limited by diffusion. Secondly, in many chemical processes a product of a reaction is less soluble in the reaction mixture than a substrate. In these cases, the precipitation of a product from the reaction mixture makes product isolation and reuse of immobilized enzyme difficult. Thus, it is highly desirable to create a catalyst which combines the advantages of both water-soluble (homogeneous) and water-insoluble (heterogeneous) catalysts.

To combine the salient features of immobilized and soluble enzymes, immobilization can be performed on so-called "smart polymers"[50] that undergo a reversible phase separation from water with small changes in the environmental parameters (pH, ionic strength or temperature). In the most effective systems, these phase transitions are fast, completely reversible and take place within a narrow range of environmental parameters. Several pH-controlled solubility systems have been developed. They include immobilization of enzymes on carboxymethylcellulose, synthetic polyelectrolytes or on polyelectrolyte complexes[51]. The drawbacks of these systems stem from the fact that precipitation normally occurs at low pH (5.5 or lower), which may lead to inactivation of many enzymes. In addition, the repeated use of acids and bases for pH adjustment leads to accumulation of salts and subsequent loss of enzyme activity or change of precipitation pattern. In this regard, temperature-sensitive polymers[52] may be advantageous if the precipitation can be achieved at temperatures near that of biological systems. One such system, based on oligomers of *N-iso* propyl acrylamide[53], has been successfully used in the laboratory for repeated solution-precipitation cycling without significant loss of activity.

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7
Reaction Engineering for Enzyme-Catalyzed Biotransformations

Manfred Biselli, Udo Kragl and Christian Wandrey

7.1
Introduction

The application of isolated enzymes to preparative organic synthesis on an industrial scale is a matter of active research worldwide. Since the late sixties, immobilized enzymes have been used in amino acid production in continuous processes on a large scale[1, 2]. In the late seventies, the use of soluble enzymes, especially in membrane reactors, broadened the scope of enzyme technology[3, 4] and opened the way to simultaneous use of more than one enzyme for complex conversions – especially coenzyme-dependent biotransformations[5–7]. In the early 1980s the use of enzymes was extended further to involve organic solvents[8, 9].

Enzymes are used as catalysts for large scale bioconversions e.g. glucose isomerase in the high fructose corn syrup (HFCS) process[10], penicillinase in the synthesis of semisynthetic penicillins[11], as well as aminoamidase[12, 13] and aminoacylase[14] in the production of L-amino acids. Additionally, a variety of processes for fine chemical synthesis has been developed, e.g. for amino acids, peptides and a broad spectrum of other optically active substances[15–23].

Based on the classical methods of enzyme isolation and characterization and the screening for appropriate microorganisms, about 3200 different enzymes are known today and are listed with E.C. numbers[15, 24]. Modern methods of genetic engineering give access to sufficient quantities of enzymes by overexpression in microorganisms, thus reducing costs of enzymes[25–28]. Enzyme reaction engineering allows further reduction of the amount of enzyme consumed per kilogram of product. Therefore, costs of enzymes do not necessarily dominate the overall cost of production, but they are often, still, a major factor. The productivity of enzyme reactors typically exceeds the level of 100 g product per litre of reactor volume per day (extreme values of 25 $kgL^{-1}d^{-1}$ are reported)[29] and consequently is no longer far below the productivity of classical chemical synthesis.

The aim of this contribution is to illustrate some basic aspects of a strategy to optimize an enzymatic process, starting from considerations focusing on the enzymes used and the reactions involved. Additional investigation of the enzyme

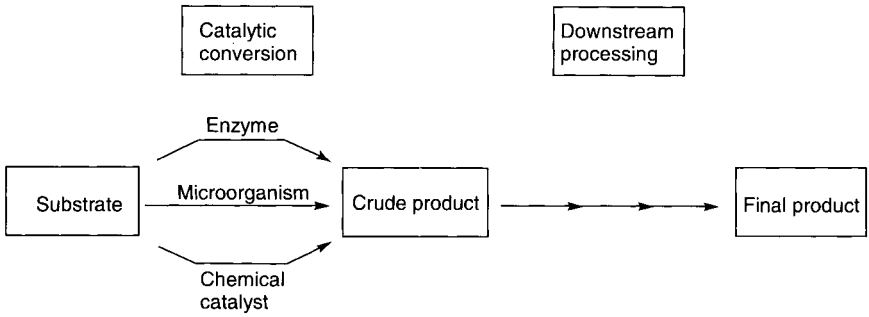


Figure 7-1. Process design.

kinetics yields a deeper insight into the process and is the basis of final optimization of process performance by reaction engineering methods. A detailed process optimization is of great significance especially for pilot and production scale.

7.2 Steps of Process Optimization

Considerations about process design precede any detailed process optimization. Process design includes the choice of an appropriate substrate, of a catalyst and of methods for downstream processing in order to gain the final product in a defined purity (Fig. 7-1).

The situation may be exemplified by showing the different catalytic methods of asymmetric synthesis of L-phenylalanine, starting from six different substrates (Fig. 7-2). Additionally, fermentation processes, using glucose as the carbon and energy source, have been developed [30–32].

Within the scope of this chapter, only enzyme-catalyzed biotransformations are considered, presuming

- a defined product (especially important in the context of this book is the demand for high optical purity of the product),
- a defined enzyme (criteria for the usefulness of an enzyme are its activity, selectivity, stability, coenzyme dependency and its kinetic constants; process optimization studies have to cover these subjects and therefore may influence the choice of the enzyme, e.g. using an enzyme from a different source),
- defined substrate(s) (the selection of a substrate including the method of enzymatic transformation is determined by availability and price of both enzyme and substrate (see Fig. 7-2).

The aim of process optimization is to find process conditions defined by

- high conversion of the substrate,
- high selectivity of the reaction,
- high optical purity of the product,

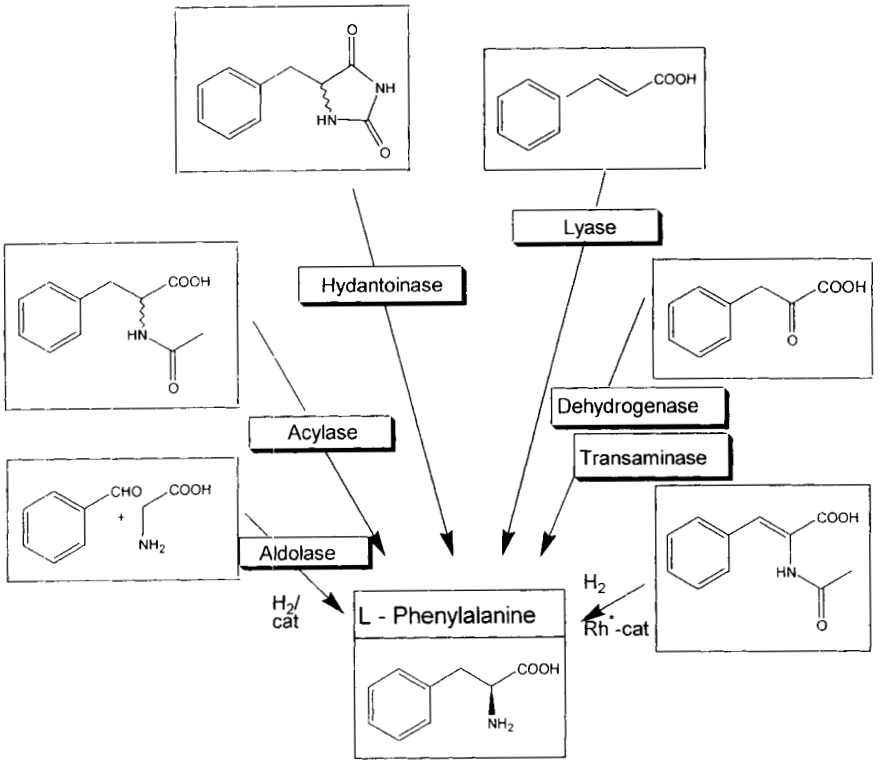


Figure 7-2. Asymmetric synthesis of L-phenylalanine.

- high space-time yield (productivity) of the process,
- low enzyme and coenzyme consumption per unit mass of product.

The overall process development basically consists of three steps (Fig. 7-3).

The ***first step*** is the investigation of the reaction system. This can be further classified into two parts:

First of all the enzyme properties have to be examined, considering the following aspects:

- reaction catalyzed,
- substrates, cosubstrates, coenzymes, effectors, inhibitors,
- dependency of *enzyme activity* on substrate concentration, temperature and pH value,
- dependency of *enzyme stability* on pH, temperature, oxidizing agents,
- dependency of *enzyme selectivity* on reaction conditions,
- necessity of immobilization for enzyme stabilization,
- considerations on the use of organic solvent/cosolvents,

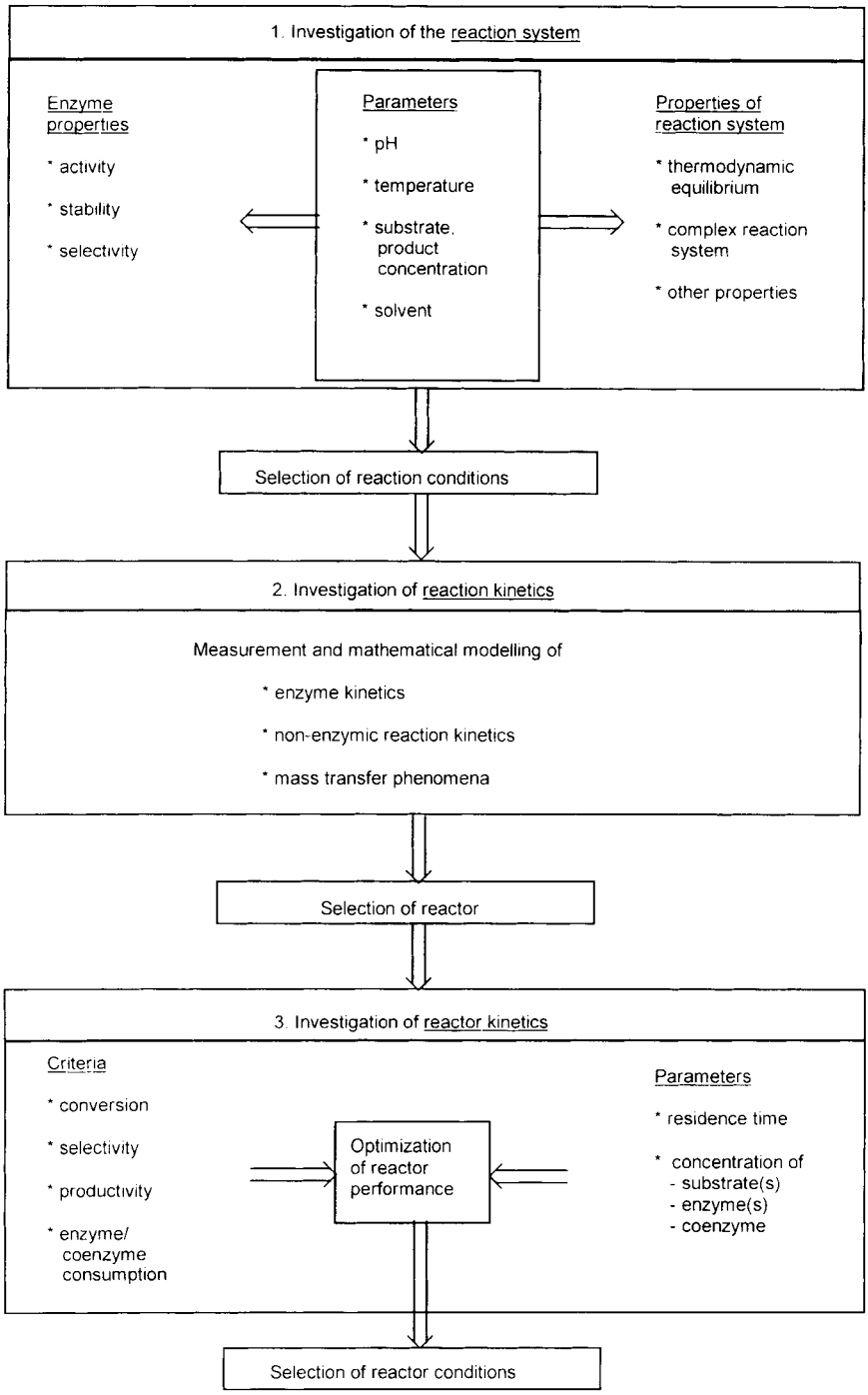


Figure 7-3. Steps of process optimization in enzyme reaction engineering.

Then the other properties of the reaction system have to be characterized. The following topics are of major concern:

- Equilibrium constant of reversible reactions,
- Structure of the reaction system. The term "structure" should include the sum of the chemical reactions occurring within the reaction system, e.g. parallel reactions, consecutive reactions (see Eq. (4)), coupled reactions such as in the case of coenzyme regeneration (see Eq. (49)), non-catalyzed reactions occurring alongside the enzymatic reaction.
- Influence of the reaction conditions (pH, temperature, concentration of substrates, organic solvent/cosolvents) on the reaction system and on the equilibrium constant,
- Other properties such as pH- and temperature effects of the reaction and solubility of substrates and products.

There are reciprocal relationships between the parameters summarized above. On the one hand enzyme stability measurements strongly depend on the concentrations of substrates, coenzymes, buffers etc. in the assay. On the other hand the choice of an appropriate concentration level is a consequence of the enzyme kinetics investigated afterwards. A compromise has to be found between different optimization criteria e.g. a lower temperature leads to a reduced enzyme activity but results in a higher enzyme stability. In the example of the oxynitrilase reaction (Eq. (12)) a low pH value is a prerequisite for high enantiomeric purity of the product but lowers enzyme activity. As a consequence, only a rough optimization can be carried out at this level.

The investigation of the reaction system ends with a (preliminary) selection of reaction conditions. This is a prerequisite for investigation of the reaction kinetics as it makes no sense to measure kinetics without knowing whether the kinetic assay conditions are favorable for the final process.

The ***second step*** in process optimization is the investigation of the reaction kinetics. This step aims at a detailed analysis of the kinetics of the reaction system under the optimized reaction conditions chosen above. The following topics have to be covered:

- Measurement and modeling of the kinetics of the enzyme-catalyzed reaction(s),
- Measurement and modeling of the kinetics of the non-enzyme-catalyzed reaction(s),
- Measurement and modeling of mass transfer phenomena. These will occur in reaction systems which consist of two or more phases, such as in the case of immobilized enzymes or in the case of liquid/liquid two-phase systems. This aspect will not be covered and the reader is referred to the literature[33–40].

The second step results in a kinetic model for the whole reaction system and a choice of an appropriate reactor based on the reaction kinetics. Criteria for reactor choice will be discussed in Sect. 7.5.1.

The ***final step*** includes the reactor design and the prediction of reactor performance depending on operation conditions. The scope of points to consider is as follows:

- Formulation of the reactor kinetics by combining the kinetic model of enzymatic and non-enzymatic reactions and mass balances of the reactor.
- Description of the reactor performance as a function of parameters such as substrate concentration, enzyme and coenzyme concentration and residence time.
- Optimization of reactor performance based on the following criteria: substrate conversion, selectivity, space-time yield, enzyme and coenzyme consumption.

The extent of optimization in the development of an enzymatic reaction may be described as follows:

- Studies with one-enzyme systems on a laboratory scale with the aim of small scale production or verification of the desired concept do not require a detailed kinetic analysis. The properties of the enzyme and of the reaction system should be investigated to choose convenient assay and reaction conditions (e.g. temperature, pH value, substrate and cosubstrate concentrations). Typically some essential enzyme data are provided by the enzyme supplier or can be taken from handbooks [41].
- Also some basic kinetic data of the enzyme should be known, especially Michaelis and inhibition constants. For example, an enzyme suffering substrate inhibition, when used at a high substrate concentration, will exhibit only a little activity, whereas at low concentrations the reaction will run quite well.
- Dealing with complex systems (two or more coupled enzymatic reactions or reactions with coenzyme regeneration) a complete kinetic investigation and computer simulation of the reaction system is very helpful to achieve the desired selectivity and yield of reaction (e.g. by choosing a sensible substrate and coenzyme concentration, enzyme ratio and reaction time). A case study is available [42, 43] exemplifying the production of L-*tert*-leucine by reductive amination and simultaneous coenzyme regeneration.
- To optimize a production process all topics listed above have to be treated in detail to achieve the optimum process performance.

7.3 Investigation of the Reaction System

7.3.1 Properties of the Enzyme

The purpose of studies on enzyme properties is to select favorable reaction conditions for the investigation of enzyme kinetics. The choice of assay conditions has to be performed very carefully, and it has to be proven that the assay conditions are as close as possible to the reactor conditions of the final process. This aspect cannot be stressed too much!

The influence of all relevant reaction parameters on enzyme *activity*, *selectivity* and *stability* has to be considered. Parameters determining the enzyme properties are

listed below (the most important of them have been discussed already in Chap. 7.1):

- Substrates, cosubstrates, coenzymes, effectors, inhibitors
- pH value
- Temperature
- Buffer
- Organic solvents/cosolvents
- Ionic strength
- Viscosity of the medium
- Enzyme modification by
  - immobilization
  - covalent modification of the enzyme
- Redox potential (oxygen sensitivity)
- Heavy metal ions
- Influences resulting from reactor conditions
  - shear stress
  - effects of the reactor material
  - surface effects (adsorption on reactor surfaces, liquid/liquid or gas/liquid interfaces).

Measurement and modeling of the influence of concentrations of substrates, cosubstrates, coenzymes and inhibitors on enzyme activity form the central subject of enzyme kinetics and are discussed in Sect. 7.4.

A few of the aspects of practical importance are as follows:

First of all the pH value and the temperature of the assay have to be chosen. The *pH-optimum of the enzyme* is determined by measuring the effect of pH on activity. It has to be recognized that the location of the pH-optimum depends on

- the substrate,
- the choice of buffer,
- the reaction medium (organic cosolvents change dissociation equilibria at the enzyme),
- the ionic strength,
- chemical derivatization or immobilization of the enzyme.

In addition the *temperature dependency of enzyme activity* must be measured, also yielding an optimum curve. This "temperature optimum" depends on the assay conditions, especially the incubation time, and is not, on its own, useful to identify a reasonable reaction temperature. Instead of this, the *temperature stability of the enzyme* has to be determined. To that end the enzyme is incubated with all relevant reaction components in test tubes, changing the temperature while keeping all other parameters constant. The assay conditions have to be as close as possible to the conditions relevant in the final process. In particular, stability measurements have to be performed in the presence of a relevant concentration of all substrates and coenzymes which have a stabilizing influence on the enzyme.

Typically, the remaining activity is plotted versus incubation time and the deactivation rate can be taken from the slope of a half logarithmic plot (Fig. 7-4).

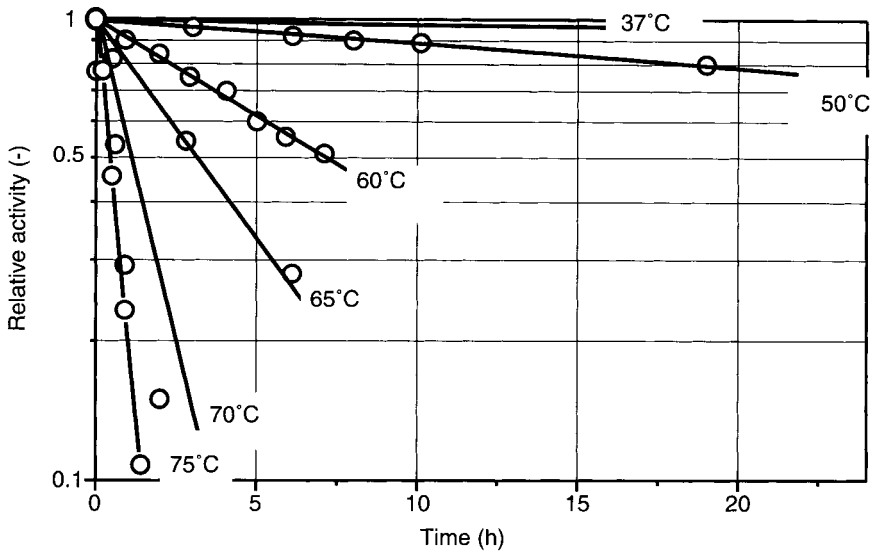


Figure 7-4. Influence of temperature on enzyme stability; example: aminoacylase[44].

The activation energy of an enzymatic reaction (typical values 20–60 kJ/mol) is far below the activation energy of thermal denaturation (values between 200 and 600 kJ/mol)[45]. Therefore, by lowering the temperature, the deactivation rate decreases more rapidly than enzyme activity and more product is obtained during the mean lifetime of the enzyme. In practice, the temperature is lowered until enzyme stability is acceptable or other denaturation effects become dominant. Lowering of reaction temperature is of course limited by the solubilities of the substrates and the freezing point of water.

Similar measurements of enzyme activity and stability have to be performed, varying the other parameters mentioned above. The *dependency of enzyme stability on pH and ionic strength* are quite important. The same method may also be used for immobilized enzymes.

In order to identify influences of the reactor operation, *enzyme stability* has to be measured under the *process conditions*[46]. Figure 7-5 demonstrates that the deactivation rate may be greatly enhanced by the conditions of reactor operation and is influenced by the reactor material.

The decrease in enzyme activity in a continuous reactor causes a decreasing conversion of the substrate. Only in the case of low conversion (compare to "initial reaction rate", see Sect. 7.4.1), the decrease of enzyme activity is proportional to the decrease of conversion and may be calculated from these data. Alternatively, the remaining activity in the reactor may be measured by taking samples of the enzyme (especially when using membrane reactors) or by adding fresh enzyme, until the initial conversion is re-established. In stirred tank reactors, the product of enzyme activity and residence time determines the conversion (see Sect. 7.5.1). Therefore,

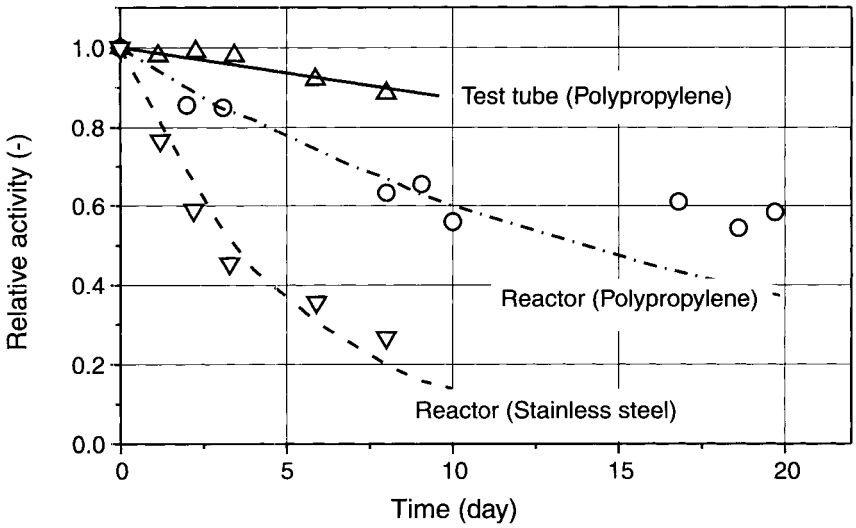


Figure 7-5. Deactivation of Neu5Ac-Aldolase depending on the reactor material[47].

the flow rate can be lowered until the initial degree of conversion is reached again and the residual enzyme activity can be calculated from the flow rates.

The question of *enzyme immobilization* typically depends on the stability of the soluble enzyme under process conditions.

Not only activity and stability but also selectivity of reactions may become the most important criterion in the selection of the reaction conditions. The impacts of additional reactions and of selectivity problems will be discussed in the following section.

7.3.2
Properties of the Reaction System

7.3.2.1
Thermodynamic Equilibrium of the Reaction

In the case of a hypothetical reaction (Eq. (1)).

$$S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} P \qquad (1)$$

Enzymes, as catalysts, accelerate the reaction rates (a kinetic factor). The forward reaction and the back reaction are accelerated to the same degree. The position of equilibrium (a thermodynamic measure), which is not influenced by the enzyme, yields information about the maximum conversion and therefore is of basic importance for process development. Two examples will serve to demonstrate this principle.

Example: Hydrolysis of acetyl-L-aminoacids by aminoacylase
Aminoacylase stereospecifically cleaves *N*-acetyl-L-aminoacids to acetate and L-aminoacid, a reaction well known for the production of optically pure L-aminoacids (Eq. (2)).

$$N\text{-acetyl-L-amino acid} \xrightleftharpoons{\text{aminoacylase}} \text{L-amino acid} + \text{acetate} \qquad (2)$$

The equilibrium conversion for hydrolysis of the acetyl-L-aminoacid depends on the initial oncentration. Only at zero concentration may one hundred percent conversion be attained (Fig. 7-6).

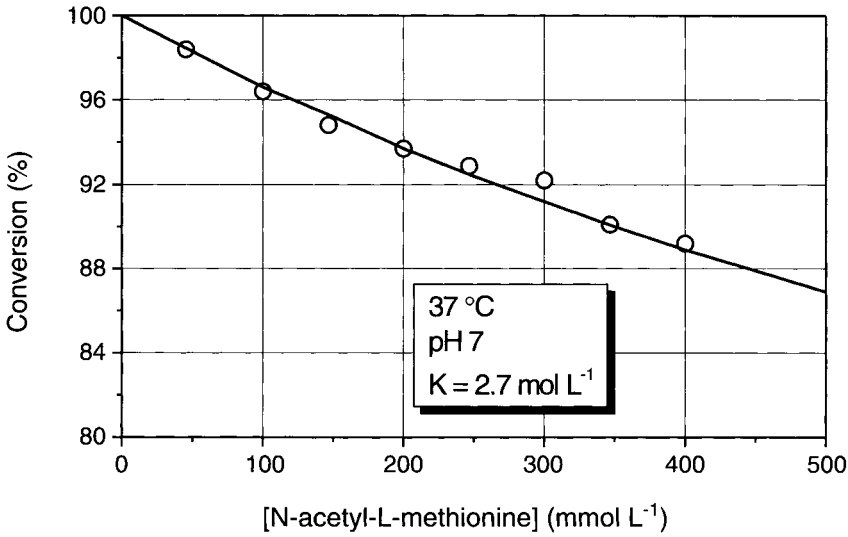


Figure 7-6. Hydrolysis of *N*-acetyl-L-methionine by aminoacylase: equilibrium conversion as function of substrate concentration [44].

In the industrial acylase process, an increase of both substrate concentration and conversion is desired to reduce costs, but, as a thermodynamic principle, in cases of increasing mole number during the reaction, the equilibrium conversion decreases with rising substrate concentration.

Example: *Synthesis of N-acetylneuraminic acid by aldolase*
N-Acetylneuraminic acid aldolase catalyzes the cleavage of *N*-acetylneuraminic acid (Neu5Ac) to *N*-acetylmannosamine (ManNAc) and pyruvate (Pyr). The reverse reaction can be employed to synthesize *N*-acetylneuraminic acid, which plays an important physiological role as a terminal sugar residue of glycosylated proteins [48] (Eq. (3)).

$$\text{ManNAc} + \text{Pyr} \xrightleftharpoons{\text{Neu5Ac-aldolase}} \text{Neu5Ac} \qquad (3)$$

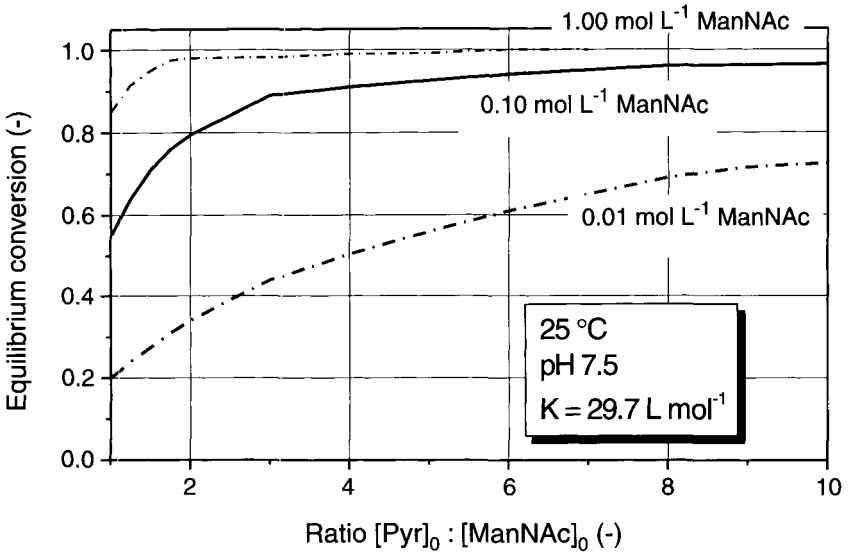


Figure 7-7. Synthesis of *N*-acetyl-neuraminic acid with Neu5Ac-aldolase: equilibrium conversion as a function of the concentration of both substrates[47].

To obtain a high conversion, high concentrations of both substrates have to be used; since ManNAc is more expensive than pyruvate, the latter is used in excess (Fig. 7-7).

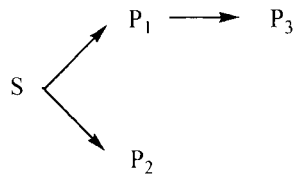
Both examples demonstrate how the maximum conversion can be influenced by the choice of appropriate substrate concentrations (without changing the equilibrium constant K_{eq}). Additionally, a disadvantageous equilibrium position can be overcome by

- continuously adding a substrate,
- continuously withdrawing a product, e.g. by crystallization, extraction, electrodialysis[49–51], complexation (aspartame process[52]),
- coupling to an irreversible reaction (see Eq. (49)),
- choosing reaction conditions where K_{eq} is changed to a more favorable value:

  K_{eq} depends on pH if acids or bases are involved in the reaction,
  K_{eq} depends on temperature,
  K_{eq} may be influenced by cosolvents[53].

7.3.2.2
Complex Reaction Systems: The Existence of Parallel and Consecutive Reactions

In this chapter, two examples demonstrate that, in addition to the desired enzyme-catalyzed conversion of the substrate S to the product P_1, other reactions have to be considered, e.g. a parallel reaction of S to P_2 or a consecutive reaction of P_1 to P_3. For a hypothetical reaction scheme, see (Eq. 4).



(4)

The following definitions are set up (all stoichiometric coefficients are set to one):
Conversion x:

$$x_S = \frac{[S]_0 - [S]}{[S]_0} \tag{5}$$

The conversion x_S of the substrate S stands for the ratio of substrate consumed to the initial substrate: ($[S]_0$-$[S] = [P_1]+[P_2]+[P_3]$).
Selectivity σ:

$$\sigma = \frac{[P_1]}{[S]_0 - [S]} \tag{6}$$

The selectivity $\sigma_{P1,S}$ is defined as the product P_1 formed in relation to the substrate S consumed.
Yield Y:

$$Y_{P_1,S} = \frac{[P_1]}{[S]_0} \tag{7}$$

The yield $Y_{P1,S}$ is defined as the ratio of the product P_1 formed to the initial substrate S_0

Sometimes, these definitions are mixed up in the literature. Eq. (8) describes the relationship between conversion, selectivity and yield:

$$Y_{P_1,S} = x_s \cdot \sigma_{P_1,S} \quad (\text{yield} = \text{conversion} \cdot \text{selectivity}) \tag{8}$$

The values of yield and selectivity become equal at quantitative conversion of the substrate. The following diagram may elucidate the relation of these three parameters (Fig. 7-8).
Additionally, the following measurements are defined:
Space-time yield:

$$STY_{P_1} = \frac{P_1}{V \cdot t} \tag{9}$$

The space-time yield STY_{P1} is the amount of product P_1 produced per reactor volume V and time with the dimension of kg $L^{-1}d^{-1}$. The space time yield is also referred to as "volumetric productivity".
Enantiomeric excess:

$$ee_{P_1} = \frac{[P_1]-[P_2]}{[P_1]+[P_2]} \tag{10}$$

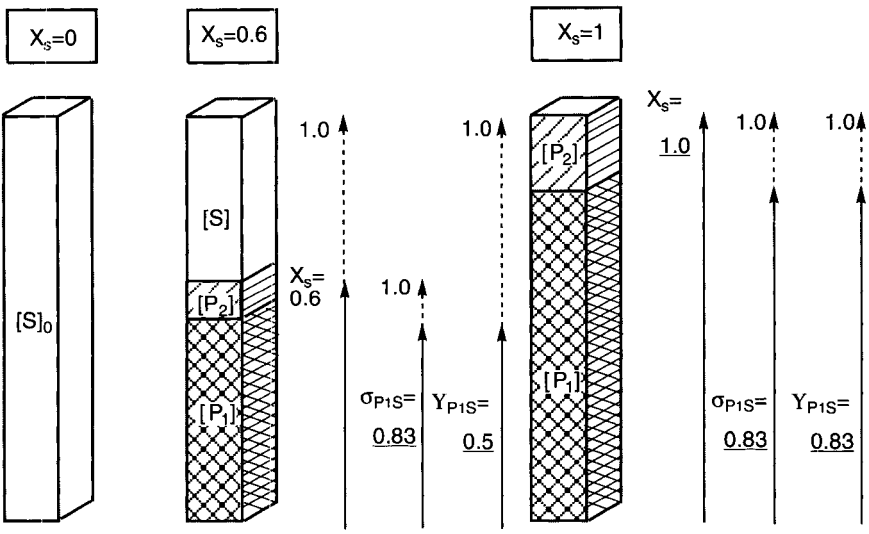


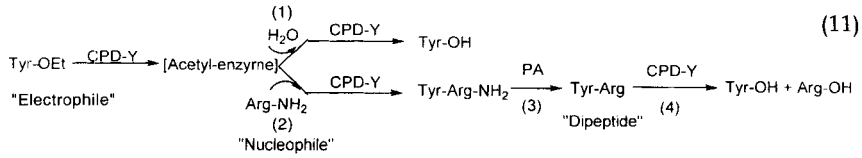
Figure 7-8. Interrelation of conversion, selectivity and yield in the case of the reaction scheme Eq. (4) (P_1 being the main product; for definitions see Eqs. (5) to (7)).

The enantiomeric excess ee_{P1} of the enantiomer P_1 is defined as surplus of P_1 in relation to the sum of enantiomers.

Kinetic parameters are defined in Sect. 7–4. Two examples will illustrate the implications of complex reactions in enzyme reaction engineering.

Example: *Enzyme-catalyzed peptide synthesis*

An instructive example of the occurrence of selectivity problems is available from the field of enzymatic peptide synthesis. For instance, the following complex reaction scheme shows where parallel reactions and consecutive reactions occur. It describes the synthesis of Tyr-Arg from the electrophile Tyr-OEt and the nucleophile Arg-NH_2 by simultaneous use of Carboxypeptidase Y (CPD-Y) and Peptide amidase (PA) from orange flavedo[54] (Eq. (11)).



(11)

The protease Carboxypeptidase Y (CPD-Y) reacts with the tyrosine ester forming an acylenzyme activated complex, which is further converted in a competitive way. Aminolysis of the acylenzyme by Arg-NH_2 (2) yields the dipeptide amide Tyr-Arg-NH_2. The competing hydrolysis (1) yields the non-activated tyrosine. The dipeptide amide Tyr-Arg-NH_2 is deamidated selectively by peptide amidase (PA) to the desired

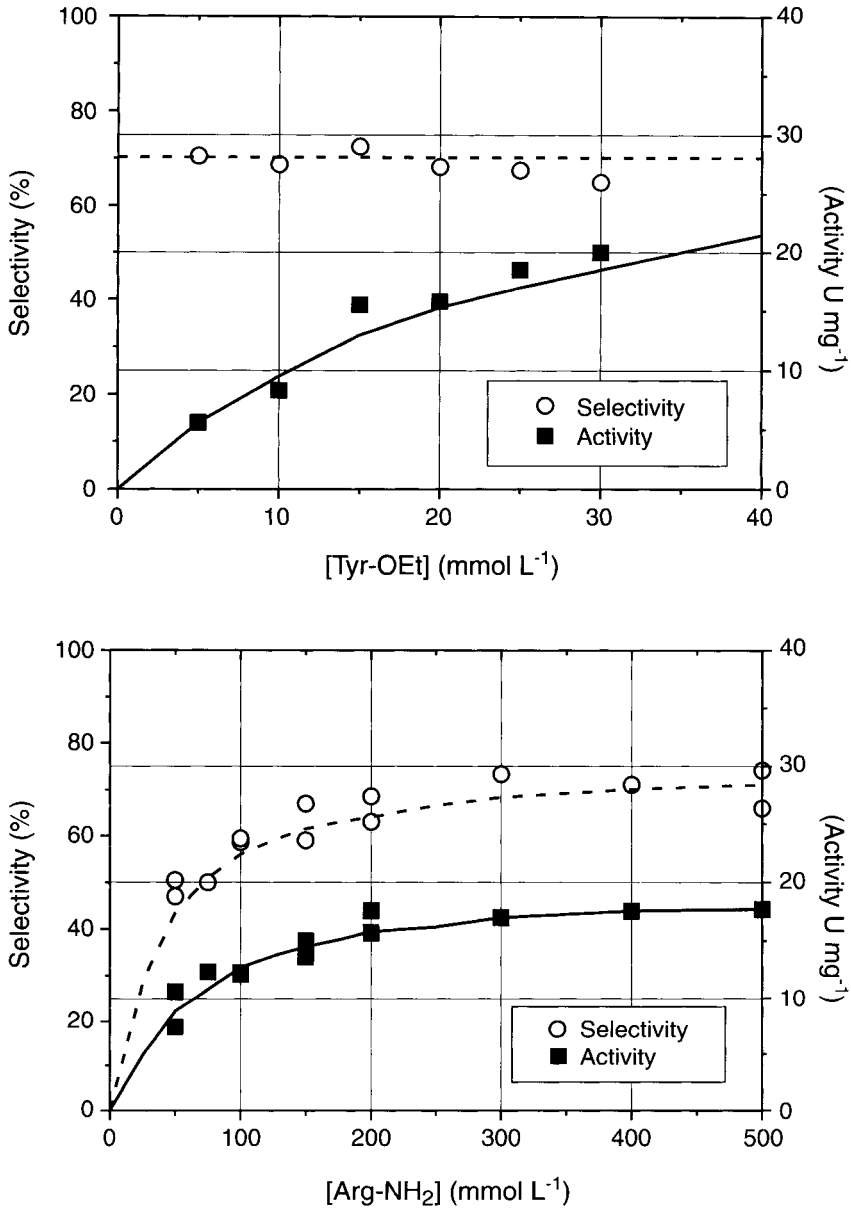


Figure 7-9 a, b. Kinetics of the synthesis of Tyr-Arg-NH_2 by CPD-Y: activity and selectivity as a function of the concentrations of Tyr-OEt (A) and Arg-NH_2 (B)[54].

dipeptide, which may be further hydrolyzed by CPD-Y to the amino acids Tyr and Arg (secondary hydrolysis). Secondary hydrolysis of Tyr-Arg-NH_2 to Tyr and Arg by CPD-Y can be neglected.

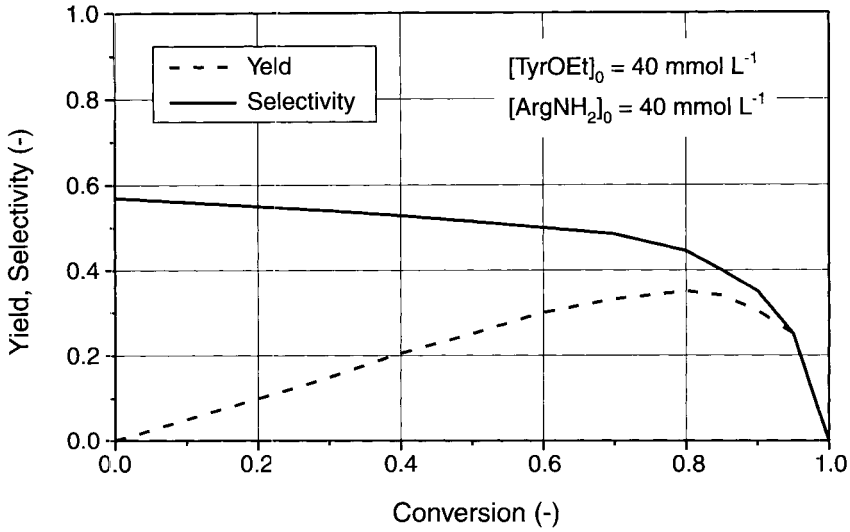


Figure 7-10. Synthesis of Tyr-Arg by CPD-Y and PA: selectivity and yield as a function of conversion for continuous operation in an enzyme membrane reactor[54].

The selectivity s of the formation of the dipeptide Tyr-Arg is reduced by the undesired parallel (1) and consecutive reaction (4). Measuring the kinetics of the aminolysis and the hydrolysis reactions gives an opportunity for process optimization (Fig. 7-9).

As expected, increasing concentration of Tyr-O-Et influences the activity of CPD-Y but not the selectivity of the competing reactions 1 and 2. On the other hand, increasing the concentration of Arg-NH_2 results in increased activity and selectivity favoring the aminolysis reaction compared to the competing hydrolysis reaction. The data in Fig. 7-9 were measured under initial rate conditions (see Sect. 7.4.1). Additionally, selectivity and yield were calculated as a function of conversion (Fig. 7-10).

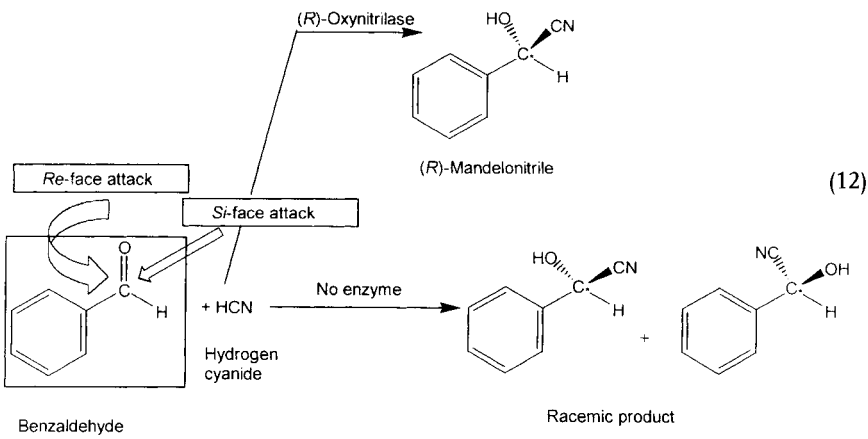
If CPD-Y and PA are used in one reactor the yield of dipeptide initially increases with conversion whereas the selectivity drops because of the consumption of the nucleophile (compare to Fig. 7-9 B). At higher conversion, the yield reaches a maximum and selectivity drops steeply because of the subsequent hydrolysis reaction (4) (Eq. (11)). At 100% conversion, yield equals selectivity, both being zero. For this reaction, a steady-state conversion of about 0.8 defines the best reactor performance. The above complex reaction is an instructive example of the correlation of selectivity, conversion and yield.

Example: *Synthesis of chiral cyanohydrins*

The "enantiomerically pure compound (EPC) synthesis" has become a major strategic concern in the synthesis of bioactive compounds especially for pharmaceutical and agrochemical use. EPC synthesis can be achieved very efficiently using

enzymes as chiral catalysts. Although enzymes mostly show a high degree of enantio- or diastereoselectivity, non-stereospecific side-reactions may occur lowering the optical purity of the product. In this case, the problem of the selectivity of the reactions has to be addressed.

A suitable catalyst for the synthesis of (*R*)-cyanohydrins is the enzyme (*R*)-oxynitrilase from bitter almonds. It catalyzes exclusively *si*-face addition of hydrogen cyanide to benzaldehyde or other aldehydes. A competing non-enzymatic parallel reaction lowers the enantiomeric excess of the product[55, 56].



(12)

This example is very instructive in that it demonstrates methods to suppress a non-desired reaction. The investigation of the reaction system leads to the question of the influence of the reaction conditions on selectivity.

Influence of pH value upon selectivity

The pH value has an influence not only on the activity of enzymes but also on chemical reactions. The chemical cyanohydrin reaction is base-catalysed, as, compared to HCN, the cyanide ion more easily attacks the carbonyl group. As a result, a distinct decrease of the reaction rate for the non-enzymatic synthesis of mandelonitrile occurs at lower pH values. Also, the enzyme activity decreases but not to the same extent; therefore, the enantioselective enzymatic reaction becomes dominant at lower pH (see Fig. 7-11).

Influence of temperature upon selectivity

The investigation of the temperature dependence of the competing parallel reactions gives information about their activation energy. According to the Arrhenius equation (Eq. (13)),

$$k = k_{\max} \cdot e^{-\frac{E_A}{RT}} \text{ or } \ln(k / k_{\max}) = -\frac{E_A}{R \cdot T} \tag{13}$$

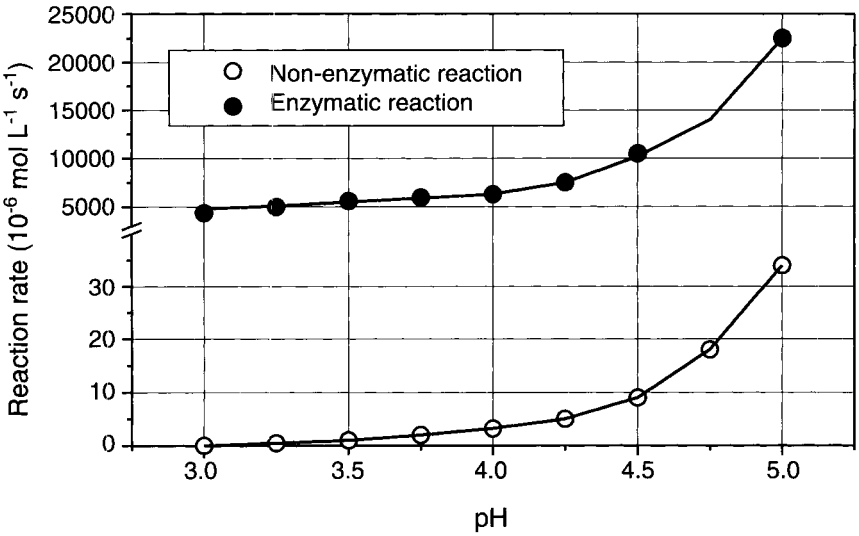


Figure 7-11. Initial reaction rate of the synthesis of mandelonitrile as function of pH [55, 56].

where

k	(s^{-1})	rate constant of the reaction
k_{max}	(s^{-1})	frequency factor
E_A	(J mol^{-1})	activation energy
R	(8.314 J $K^{-1}mol^{-1}$)	gas constant
T	(K)	absolute temperature,

the activation energy E_A can be determined from the following plot:

In this case the non-enzymatic reaction, having an activation energy of 73 kJ mol^{-1}, decreases faster with temperature than the enzymatic reaction (activation energy 46 kJ mol^{-1}). In general, at a lower temperature the reaction with the lower activation energy is favored. The enzymatic reaction therefore becomes more dominant at lower temperature.

Influence of substrate concentration

With respect to benzaldehyde, (*R*)-oxynitrilase exhibits saturation kinetics (Michaelis Menten kinetics, see Sect. 7.4.2.1) and a maximum reaction rate is reached above a concentration of about 5 mmol L^{-1}. The chemical reaction presents a linear increase of the reaction rate with increasing benzaldehyde concentration, representing first order kinetics, when the concentration of HCN is kept constant (see Fig. 7-13). As a consequence the enzymatic reaction becomes more dominating at lower concentrations of the substrate benzaldehyde (for HCN as substrate the same kinetic behavior occurs, data not shown). Accordingly an enzyme reactor would be suitable that works under minimum average substrate concentrations. These requirements are satisfied by the continuous stirred tank reactor (CSTR). In Sect. 7.5.2.1 this aspect of enzyme reaction engineering will be discussed further.

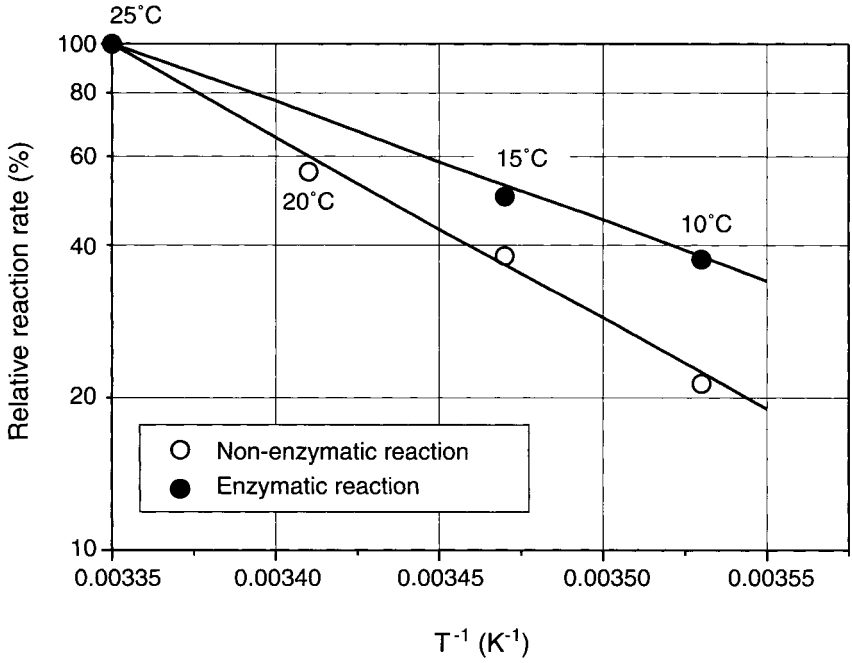


Figure 7-12. Relative reaction rate of the synthesis of mandelonitrile as a function of the reciprocal temperature[57].

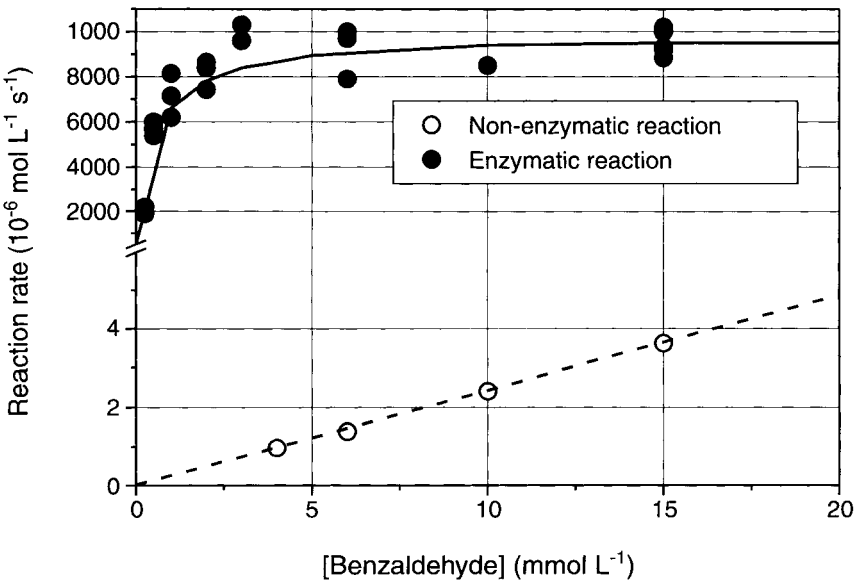


Figure 7-13. Initial reaction rate of synthesis of mandelonitrile as function of benzaldehyde concentration[55].

Influence of organic solvents
There are two arguments for the use of organic solvents in the synthesis of *alpha*-hydroxynitriles by oxynitrilase:

- the best substrates of the enzyme are aromatic and straight chain aliphatic aldehydes, both of which have a limited water solubility (e.g. benzaldehyde 80 mmol L^{-1}). The solubility may be enhanced by organic solvents;
- the non-specific reaction is favored in water and may be suppressed by lowering the water activity of the medium.

Different methods have been used, employing organic solvents in this reaction (reviewed in [58, 59]):

- the use of water-miscible organic cosolvents such as ethanol or methanol,
- the application of aqueous two-phase systems,
- immobilization of the enzyme and the use of organic solvents (e.g. ethyl acetate or others) which contain only traces of water to preserve the enzyme's activity,
- the application of biphasic lyotropic liquid crystal systems.

In the oxynitrilase reaction, high optical purities can be reached by the use of organic solvents. Often a major drawback of these methods is a lowered enzyme activity, resulting in long reaction times and a reduced enzyme stability in continuous experiments.

Selection of reaction conditions
Summarizing the above effects, the high selectivity toward the enzyme-catalyzed reaction and suppression of the non-specific parallel reaction is achieved by

- *low pH,*
- *low temperature,*
- *low stationary substrate concentrations,*
- additionally a *high enzyme activity* exclusively favors the enzyme-catalyzed reaction without influencing the non-enzymatic reaction.

Knowing that the enzyme stability is sufficiently high at pH 3.75 (50% deactivation after 150 h), water was chosen as the reaction medium and the reaction was performed at room temperature. This *selection of reaction conditions* was followed by a detailed kinetic analysis of the system, the investigation of the reactor kinetics and the simulation of steady state conditions in continuous experiments [55, 60].

The discussion about the choice of an appropriate reactor and optimized operation conditions will be continued in Sect. 7.5.2.1. Anticipating the results, the reaction can be performed effectively under the above reaction conditions in an enzyme membrane reactor yielding a product with an enantiomeric purity of higher than 99%.

7.3.2.3
Other Properties of the Reaction System

Other properties that have to be considered in enzyme reaction engineering are:

pH effects on the reaction
A pH effect during the reaction occurs if proton shifts are involved, as is the case in most enzymatic reactions. As enzyme activity is strongly dependent on pH, a pH shift induced by the reaction has to be prevented by buffering or titrating the reaction medium. Problems arise especially in immobilized systems where pH gradients occur within the enzyme matrix if the buffering capacity of the medium is too low[61, 62]. In cofactor dependent reactions the influence of pH on the stability of $NAD(P)^+$ and NAD(P)H has to be considered[63].

The effect of temperature on the reaction
The effects of the variation of temperature on enzymatic reactions usually do not require precautions, as in typical small scale reactors the capacity of heat exchangers is sufficient to maintain a constant temperature.

Solubilities of substrates and products
This information may be useful in cases where a product can be removed continuously from the reaction mixture by crystallization.

7.3.2.4
Application of Organic Solvents

The use of enzymes in non-aqueous solvents can be an advantageous alternative to reactions in water, especially for poorly water-soluble substrates and products, e.g. in the synthesis of esters, lactones or selected peptides. However, the knowledge of how solvents influence enzyme activity and selectivity is still not profound. As it is impossible to cover all relevant aspects within this chapter, the reader is referred to some selected articles for further reading[15, 64–75]. Here some of the basics will be discussed briefly; in Sect. 7.5.4 reactor concepts for the use of enzymes in organic solvents will be presented.

There are advantages in performing enzymatic transformations in non-aqueous media:

- non-specific side-reactions such as the chemical addition of HCN to aldehydes during the oxynitrilase reaction may be suppressed by performing the reaction in an organic solvent. This example has been presented in the previous section.
- the solubility of poorly water-soluble substrates or products may be increased in organic solvents or at least by addition of water-miscible solvents. If non-water-miscible solvents are used in combination with water, inhibitory effects such as substrate or product inhibition may be overcome if the organic phase contains most of the substrates and products and the enzyme remains in the water phase.

A very interesting alternative has been published detailing the use of cyclodextrins to enhance the solubility of 2-acetylnaphthalene in aqueous solution. The complex between the cyclodextrin derivative and the ketone is transformed fast enough to support high reaction rates in an enzymatic reduction [76].

- equilibrium reactions may be shifted toward the desired product for one or both of the following reasons: (i) lowering of water activity by adding a water-miscible organic solvent (example: enzymatic peptide synthesis [53]); (ii) continuous extraction of the desired product into a non-water-miscible organic phase (compare to the multiple-compartment enzyme membrane reactor (see later, Fig. 7-37, [77, 78]).

Both stabilizing and destabilizing effects of solvents on enzymes have been reported. A reasonably reliable measure of the compatibility of solvents with enzymes is the log *P* value, where *P* is defined as the distribution coefficient of a solvent between water and 1-octanol in a two-phase system [64, 79]. Solvents with a log *P* value above 4 are suitable (e.g. aromatics, aliphatics) whereas water-miscible solvents with a log *P* value below 2 (short chain esters, DMF, short-chain alcohols) are not suitable for employment with biocatalysts. The latter solvents interfere with the water at the boundary of the protein itself and so disrupt the binding forces necessary to maintain an active form of the enzyme. Surprisingly, *tert*-butanol has a stabilizing effect on some oxidative enzymes [80], despite its low log P value (0.35).

Fig. 7-14 shows a classification for biotransformations in organic solvents into one-phase and two-phase systems.

One-phase systems may consist mainly of water, water plus a water-miscible solvent or a pure organic solvent. Most *water-miscible solvents* may be used in concentrations up to 20% before enzyme deactivation exceeds the benefits obtained by better solubility of substrates and/or increased selectivity. Using this approach, enzyme activity and enzyme stability have to be examined carefully to select appropriate reaction conditions.

Using mainly *water non-miscible solvents* several approaches are possible. In most cases, the organic solvent has to be saturated with water in order not to remove the boundary water surrounding the enzyme, which otherwise results in deactivation. In such microaqueous systems the pH of this tiny amount of water should be carefully chosen for optimal enzyme activity. The control of water activity can be achieved by addition of salts or utilization of saturated salt solutions [81, 82]. The simplest way of using an enzyme in organic solvents is to suspend the insoluble enzyme in the required solvent. This technique was first reported in 1900 [83] and has been extended over the last few years to encompass many systems (mainly proteases and lipases) [75, 84, 85]. Organic solvents may be replaced by supercritical liquid carbon dioxide, which exhibits similar properties to hexane [86, 146].

To achieve true homogeneous catalysis, enzyme solubility may be increased by coupling polyethyleneglycol to its surface [87]. The coupling may alter the stability, activity and selectivity of the enzyme.

By use of detergents and small amounts of water or buffer, reversed micelles can be formed containing the enzyme in the water phase while the organic solvent forms the bulk phase [88, 89].

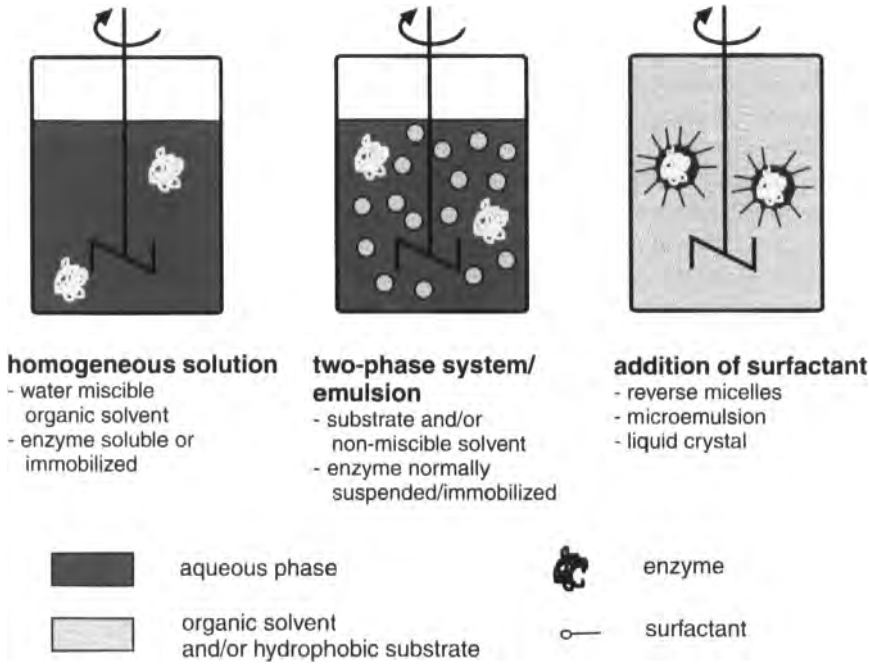


Figure 7-14. Classification of enzyme catalysis in the presence of organic solvents.

Just like one-phase systems, *two-phase systems* may consist mainly of water or mainly of organic solvent. In an aqueous-based system an insoluble substrate is dispersed, using a non-miscible solvent (oil-in-water emulsion). Hydrolysis reactions of poorly water soluble substrates (e.g. fat hydrolysis by esterases) may be performed in this way. If the aqueous phase is dispersed in a water-immiscible organic solvent a water-in-oil emulsion is obtained. This type of system may be advantageous if a condensation reaction has to be performed where the water content has to be kept low. A special form of a two-phase system involves lyotropic liquid crystals, which are obtained by mixing a detergent, water and an organic solvent[90, 91]. Most of the water and the detergent form the liquid crystal wherein the enzyme is immobilized, whereas most of the organic solvent forms the second phase.

Enzymes immobilized on an insoluble support also belong to two-phase systems. They have been mentioned earlier and are not discussed further at this point.

Very recently ionic liquids emerged as a new class of solvents. They are salts with melting points below 100 °C and are non-volatile. Some typical structures are shown in Fig. 7-15. They possess good solvating properties for many substrates and catalysts[92–94]. They can also be used to replace organic solvents in enzymatic reactions[95–98]. For the lipase-catalyzed kinetic resolution of phenylethanol an improved enantioselectivity at higher temperatures was observed compared to the selectivity obtained when using methyl-*tert*-butylether (MTBE) as solvent[97, 98].

What has been neglected so far is a consideration of the location of the enzyme

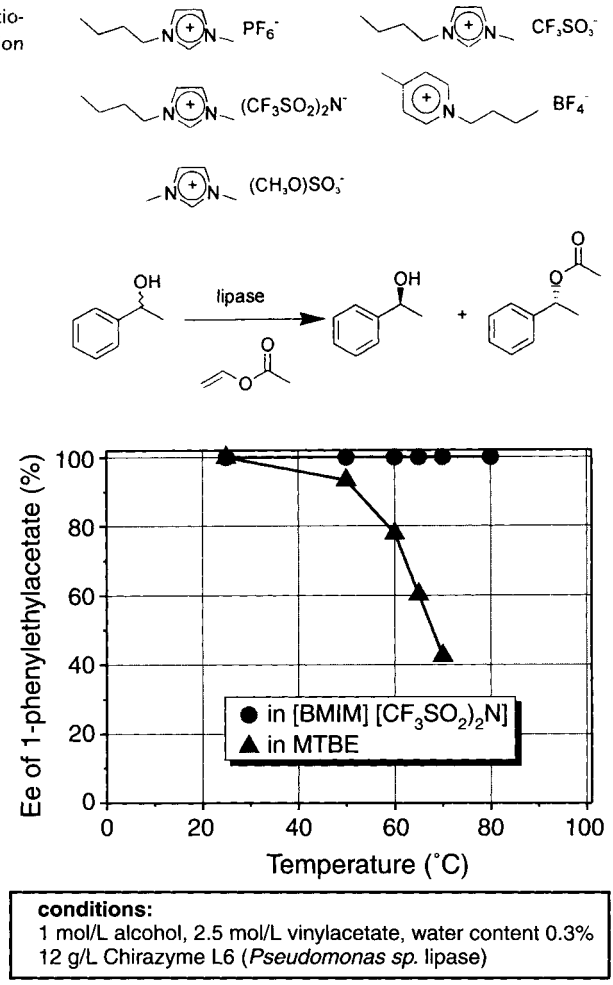


Figure 7-15. Increased enantioselectivity for lipases by reaction in ionic liquids.

and hence the enzymatic reaction. Depending on the hydrophobic and hydrophilic properties of the enzyme it may be enriched in one of the phases (normally the aqueous one). For lipases, catalysis often occurs at interfaces. Therefore, the surface area per unit reactor volume available in such a system is an important quantity, not only for reaction performance but also for determination of kinetic data.

As shown in Fig. 7-16, a larger surface area requires a higher enzyme concentration to be saturated. But a tenfold increase in the surface area – even if saturated with enzyme – does not result in a tenfold increase of conversion[99]. One reason for this might be product inhibition.

Whereas kinetic studies are quite easy to perform in homogeneous solution the extent of the interface has to be taken into consideration for biphasic systems. The most reliable way of measuring the interfacial area is by use of Fraunhofer

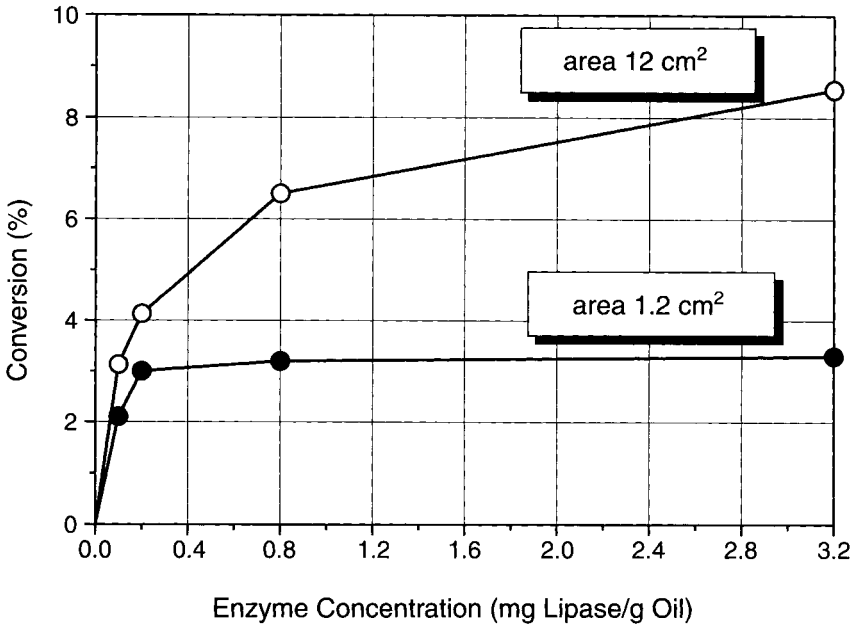


Figure 7-16. Saturation of the phase boundary surface with lipase.

diffraction[100]. All reaction velocities can be given based on the same surface area. A change of droplet size and surface area, which may occur with change of substrate or product concentration during the reaction, can be distinguished from true inhibition effects.

7.4 Investigation of Enzyme Kinetics

In this chapter, some principles of the kinetics of enzymatic reactions are discussed. A more detailed description of enzyme kinetics is covered in a number of textbooks and articles[45, 101–104]. First of all, a few general definitions will be given.

The *reaction rate* υ of any chemical reaction (Eq. (14))

$$n_A A + n_B B \rightarrow n_C C + n_D D \tag{14}$$

is defined as follows (Eq. (15)):

$$\upsilon = -\frac{1}{n_A} \cdot \frac{d[A]}{dt} = \text{function of } k, [A], [B], \ldots \tag{15}$$

υ	(mmol L^{-1} min^{-1})	reaction rate
[A], [B]	(mmol L^{-1})	concentrations of A, B ...
nA, nB	(–)	stoichiometric coefficients
k	(mmol^{1-n} L^{n-1} min^{-1})	rate constant (n being the order of reaction)

Eq. (15) is the *rate equation* of the reaction (also called the "*kinetic model*"). The formulation of such a differential equation for all reacting substances is the basic step in describing the kinetics of chemical/biochemical reactions. These rate equations include concentration values of the relevant reaction partners and kinetic parameters such as the rate constant k. An investigation of enzyme kinetics includes the measurement of reaction rates, the choosing of an appropriate kinetic model and the identification of the kinetic parameters.

7.4.1
Methods of Parameter Identification

Kinetic measurements[105] have to be carried out to examine the dependence of the reaction rate on the concentrations of all relevant components. As described in a previous chapter, for measuring enzyme kinetics *initial reaction rates* $v_0 = f[S]$ are determined at optimal reaction conditions, which may be chosen according to the procedure outlined in Sect. 7.3. The initial reaction rates are measured by varying the concentration of only one component and keeping all other concentrations (e.g. of cosubstrates and inhibitors) at a constant level (for an example, see Figs. 7-19 and 7-20). The rate of conversion has to be smaller than 5–10%, essentially to keep all initial concentration values constant.

The parameters of the kinetic model can be identified by fitting the kinetic data using methods of *non-linear regression* such as those described by Rosenbrock or Nelder Mead[106, 107] (Fig. 7-17 A). Methods of *linear regression* that are often used need a rearrangement of rate equations into a linear form (e.g. a double reciprocal plot according to Lineweaver-Burk[108]). This gives different weight to the data points measured at different concentration levels[109, 110]. For the correct calculation of the regression line this point must be considered, otherwise the Lineweaver-Burk double reciprocal plot is not acceptable[111].

Initial rates are not significant in large-scale processes where high conversion of the substrate is desired. With rising conversion, the simultaneous effects of both substrate S and product P on the reaction rate have to be described. In the case of equilibrium reactions, the forward reaction and the back reaction have to be described by one rate equation: they can only be treated separately under initial rate conditions. *The overall rate equation has to describe the reaction rate as a function of all relevant components at all relevant concentration levels.* A correct fit of all initial reaction rate data gives no guarantee that the kinetic model will fit the overall reaction data!

A proper fit of the time-courses of some batch reactor experiments at different starting concentrations represents an appropriate test of the rate equation. This implies that numerical integration of the rate equation (e.g. by the Runge Kutta method[112]), yielding a simulated time-course, has to fit the data of the measured time-course over the whole range of conversion (compare to Fig. 7-17 B). Examples of these methods will be given after the presentation of the basic kinetic models.

A combination of the Runge Kutta method and methods of non-linear regression allows a parameter identification from the time-course data. This technique starts with a given set of parameters, performs the numeric integration of the rate equation

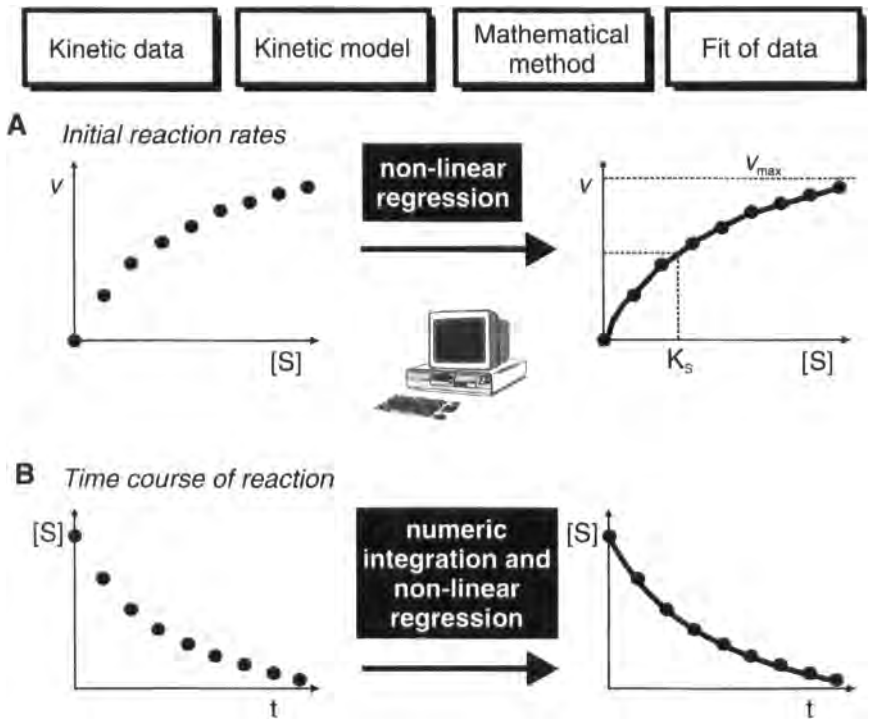


Figure 7-17. Methods of parameter identification: **A** by fitting initial rate kinetic data, and **B** by fitting the time-course of a reaction.

and compares the simulated with the measured time-course. Then the parameters are changed and the same steps are repeated until the simulation fits the measured data (Fig. 7-17 B). This method requires specially designed computer software, which is commercially available (Scientist™, Mathlab™, Maple™). The method is especially useful if components having an effect on the enzyme kinetics are not available or if they cannot be measured, but are in a defined stoichiometric relation to a measurable component.

7.4.2
The Kinetics of One-Enzyme Systems

7.4.2.1
Michaelis-Menten kinetics

In this section the basic kinetic model for enzyme-catalyzed bioconversions is presented. Understanding this model is the foundation for deriving more complex models. In their theory of enzyme catalysis, Michaelis and Menten [113] postulated the existence of an enzyme substrate complex (ES), which is built up in a reversible

reaction of the substrate S and the enzyme E. The dissociation of this ES complex to E and P is assumed to be the rate-determining step (Eq. (16)).

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P \tag{16}$$

Rate constant		Reaction
k_1	($L\ mmol^{-1}min^{-1}$)	Association of the ES complex
k_{-1}	(min^{-1})	Dissociation of the ES complex into E and S
k_2	(min^{-1})	"Turnover number" (see below) (also refered as "k_{cat}")

If the rate constant k_2 is much smaller than the rate constant k_{-1} of the enzyme, the substrate and the enzyme-substrate complex are in equilibrium, which is not disturbed by the decomposition of ES into E and P (*"rapid equilibrium-assumption"*). Based on this assumption, Michaelis and Menten derived the following rate equation (Eq. (17)):

$$\upsilon = \frac{\upsilon_{max} \cdot [S]}{K_S + [S]} \tag{17}$$

υ	($mmol\ L^{-1}min^{-1}$)	Reaction rate
υ_{max}	($mmol\ L^{-1}min^{-1}$)	Maximum reaction rate
K_S	($mmol\ L^{-1}$)	Dissociation constant of the ES complex

$$K_S = \frac{k_{-1}}{k_1}$$

This is the classic form of the Michaelis-Menten equation. Eq. (17) can be rearranged to

$$\upsilon = \frac{\frac{\upsilon_{max}}{K_S} \cdot [S]}{1 + \frac{[S]}{K_S}} \tag{18}$$

$\frac{\upsilon_{max}}{K_S}$	(min^{-1})	represents the rate constant of the first order enzyme kinetics (see Fig. 7-18)
$1 + \frac{[S]}{K_S}$	(-)	denominator of the kinetic model : represents a dimensionless "*adsorption term*" (see below)
$\frac{[S]}{K_S} = \frac{[ES]}{[E]}$	(-)	represents the dissociation of ES into E and S and may be simply derived from the dissociation equilibrium

The curve $v = f[S]$ belonging to the Michaelis-Menten function is shown in Fig. 7-18.

As long as [S] is well below K_S' Michaelis-Menten kinetics transpose to (linear) first-order kinetics. In this case nearly all enzyme molecules are present in the free form and saturation of the enzyme with substrate is rate limiting. This degree of saturation is represented by the *"adsorption term"*, which gives the sum of the values 1 + [ES]/[E] + [ES*i*]/[E] (if more substrates S_i or inhibitors or products are involved). *The adsorption term represents the dissociation equilibria of all relevant enzyme-substrate*

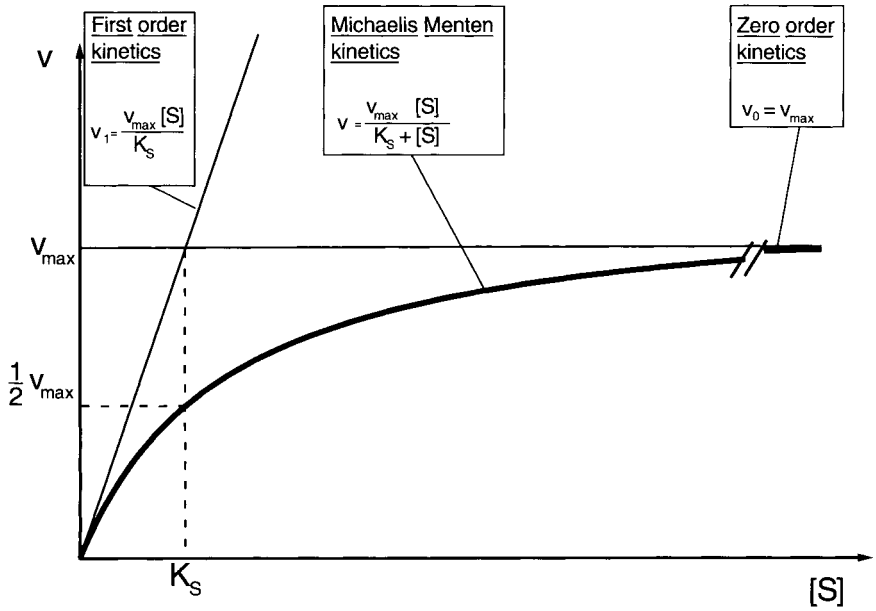


Figure 7-18. Michaelis-Menten kinetics: curve analysis.

complexes, and reveals the "weight" of the equilibria involved. For example, for [S] = K_S the value of the adsorption term equals 2, so that 50% of the enzyme is in the ES form and the other 50% is in the free form. The adsorption term initially grows from a value of 1 (at [S] << K_S) slowly to become proportional to [S] and transforms the linear first-order rate law to the curve reflecting Michaelis-Menten kinetics.

At concentration values above K_S it is not enzyme saturation but the catalytic turnover of the enzyme that becomes more and more limiting. Being proportional to the concentration of the ES complex, the reaction rate cannot rise above the v_{max} value when all enzyme molecules are in the ES form. The enzyme kinetics turn to zero order.

The Michaelis-Menten kinetics, represented by Eq. (18), may be extended to more complicated reactions by looking at the structure of the adsorption term. This procedure shown below is valid as long as the "rapid equilibrium" assumption is made. This is not valid in all cases.

Briggs and Haldane[114] did not use the above assumption, but pointed out that within a very short time after starting the reaction, ES would build up to a nearly "steady state" level where the following assumption is valid: d[ES]/dt = 0. From this "*steady state assumption*" they derived the same rate equation:

$$\upsilon = \frac{\upsilon_{max} \cdot [S]}{K_M + [S]} \qquad \text{with } K_M = \frac{k_{-1} + k_2}{k_1} \tag{19}$$

K_M (mmol L^{-1}) Michaelis constant

Here the K_M value no longer is a *dissociation constant* but a *kinetic constant* which includes k_1, k_{-1}, k_2 and for more complex reactions even more rate constants. This equation is the standard form of the Michaelis-Menten equation.

Although the derivation of reaction rates using the *"steady state assumption"* is more exact, often the rapid equilibrium assumption is used because it allows a simple derivation of the rate equation from the relevant enzyme-substrate complexes (see below) and allows fitting of the kinetic data. The following explanations are based on the rapid equilibrium assumption, and therefore *all following constants K are used as dissociation constants with the component dissociating from the enzyme as the subscript, e.g.* K_A, K_B, *and the component remaining at the enzyme as second subscript (e.g.* K_I^S, *see below).*

Within Eq. (17) υ_{max} can be expressed as shown in Eq. (20):

$$\upsilon_{max} = [E]_0 \cdot k_2 \tag{20}$$

υ_{max}	(mmol $L^{-1}min^{-1}$)	Maximum reaction rate
$[E]_o'$	(mmol L^{-1})	Total enzyme conzentration in molar value: $[E]_o' = [E] + [ES]$
k_2	(min^{-1})	Turnover number

The *"turnover number"* represents the moles of product formed per minute and per mole of active sites of enzyme. Usually the molar concentration of the active sites is not known, so the weight per volume of the enzyme preparation is used; Eq. (20) is changed to Eq. (21).

$$\upsilon_{max} = [E]_0 \cdot A_{max} \tag{21}$$

$[E]_o$	(g L^{-1})	Total enzyme concentration (in g L^{-1})
A_{max}	(mmol g^{-1} min^{-1})	Maximum specific activity of the enzyme

Using Eq. (21), Eq.(17) can be converted to Eq. (22).

$$\upsilon = [E]_0 \cdot \frac{A_{max} \cdot [S]}{K_S + [S]} \tag{22}$$

and, with Eq. (23),

$$A = \frac{\upsilon}{[E]_0} \tag{23}$$

A	(mmol g^{-1} min^1)	Specific activity of the enzyme

to Eq. (24).

$$A = \frac{A_{max} \cdot [S]}{K_S + [S]} \tag{24}$$

Besides specific activity, two other quantities are used for the characterization of enzymes: the *International Unit [U]* (defined as the amount of enzyme which

produces 1 μmol of product per minute) and the *katal (kat)* (defined as the amount of enzyme which produces 1 mol of product per second).

The following correlations exist between the reaction rate v, the specific activity A and the definitions of unit U and katal (kat)(Eq. (25)):

$$\begin{aligned} &1\frac{\mathrm{U}}{\mathrm{mL}} = 1\frac{\mu\,\mathrm{mol}}{\mathrm{mL}\cdot\mathrm{min}} = 1\frac{\mathrm{mmol}}{\mathrm{L}\cdot\mathrm{min}} && \text{(reaction rate = volumetric activity)} \\ &1\frac{\mathrm{U}}{\mathrm{mg}} = 1\frac{\mu\,\mathrm{mol}}{\mathrm{mg}\cdot\mathrm{min}} = 1\frac{\mathrm{mmol}}{\mathrm{g}\cdot\mathrm{min}} && \text{(specific activity)} \\ &1\,\mathrm{kat} = 6\cdot 10^{7}\,\mathrm{U} && \text{(activity)} \end{aligned} \tag{25}$$

The use of values for the specific activity of an enzyme is only significant if the conditions of measurement are specified, especially temperature, substrate concentration and pH value. Additionally, enzyme activities are always measured at initial reaction rate conditions (see above).

In the following sections the extension of Eq. (18) to more complex reaction schemes is described. Again the *"rapid equilibrium assumption"* is used to show how more complex rate equations are derived from simple Michaelis-Menten kinetics. Attention is focused on some typical rate equations that are useful to describe enzyme kinetics with respect to a desired process optimization. The whole complexity of enzyme kinetics is of importance for a basic understanding of the enzyme mechanism, but it is not necessary for the fitting of kinetic data and the calculation of reactor performance.

7.4.2.2
Competitive Inhibition

Substances that cannot be converted by the enzyme but are competing with the substrate for the active site of the enzyme are called *"competitive inhibitors"*. The following reaction scheme represents this situation:

$$\begin{array}{ccccc} \mathrm{E} + \mathrm{S} & \overset{K_S}{\rightleftharpoons} & \mathrm{ES} & \xrightarrow{k_2} & \mathrm{E} + \mathrm{P} \\ + & & & & \\ \mathrm{I} & & & & \\ \updownarrow K_I & & & & \\ \mathrm{EI} & & & & \end{array} \tag{26}$$

I		Inhibitor
K_I	(mmol L^{-1})	Dissociation constant of the EI-complex

The corresponding kinetic model again may be presented in two forms (Eqs. (27 and 28)).

$$v = \frac{\frac{v_{max}}{K_S}\cdot[\mathrm{S}]}{1 + \frac{[\mathrm{S}]}{K_S} + \frac{[\mathrm{I}]}{K_I}} \tag{27}$$

$$\upsilon = \frac{\upsilon_{max} \cdot [S]}{K_S \cdot \left(1 + \frac{[I]}{K_I}\right) + [S]} \tag{28}$$

Compared to Eq. (18) the rate equation (Eq. (27)) for competitive inhibitors includes an additional term $[I]/K_I$ in the denominator representing the additional dissociation equilibrium of the EI complex (*cf.* the above discussion about the "*adsorption term*"). Also, each alternative substrate S_n of a reaction would render such a term $[Sn]/K_{Sn}$ in the denominator. As a consequence of their affinity to the enzyme, alternative substrates and inhibitors block a part of the enzyme otherwise available for the reaction $S \rightarrow P$.

Thus, the product P of enzymatic reactions is often a competitive inhibitor of the enzyme leading to "*product inhibition*" (compare Eqs. (27) and (37)). The influence of product in the case of reversible reactions will be discussed later.

From Eq. (28) it can be concluded that the effect of a competitive inhibitor is to increase the apparent K_S value while the υ_{max} value is not affected. For example if [I] is chosen as K_I, in the presence of the inhibitor it would take twice as much substrate S to reach $\upsilon_{max}/2$ as without inhibitor.

7.4.2.3
Non-Competitive Inhibition

"*Non-competitive inhibitors*" bind at the enzyme E or at the ES complex and build up a ternary complex ESI, which cannot be converted to the product P (Eq. (29)):

$$\begin{array}{ccccc} E + S & \overset{K_S}{\rightleftharpoons} & ES & \xrightarrow{k_2} & E + P \\ + & & + & & \\ I & & I & & \\ \Big\updownarrow K_I & & \Big\updownarrow K_I^S & & \\ EI + S & \overset{K_S^I}{\rightleftharpoons} & ESI & & \end{array} \tag{29}$$

K_I	(mmol L^{-1})	Dissociation constant of the EI-complex with release of I
$K_I S$	(mmol L^{-1})	Dissociation constant of the ESI-complex with release of I
$K_S I$	(mmol L^{-1})	Dissociation constant of the ESI-complex with release of S

For ordinary non-competitive inhibition, the K_I and K_I^S values are identical and also the K_S and K_S^I values. In this case rate equation (30) is valid:

$$\upsilon = \frac{\frac{\upsilon_{max}}{K_S} \cdot [S]}{1 + \frac{[S]}{K_S} + \frac{[I]}{K_I} + \frac{[S] \cdot [I]}{K_S \cdot K_I}} \tag{30}$$

$$\upsilon = \frac{\upsilon_{max} \cdot [S]}{\left(1 + \frac{[I]}{K_I}\right) \cdot (K_S + [S])} \tag{31}$$

Compared to Eq. (27), the rate equation for non-competitive inhibitor includes another term for the equilibrium of decomposition of the ESI-complex into E, S and I.

From Eq. (31) it can be concluded that a non-competitive inhibitor has no effect on the K_S value but lowers the υ_{max} value. This is because the inhibitor binds all enzyme species with the same affinity.

7.4.2.4
Uncompetitive Inhibition

Substances which only bind the ES complex and not the free enzyme E are "*uncompetitive inhibitors*":

$$\begin{array}{ccccc} E + S & \overset{K_S}{\rightleftharpoons} & ES & \xrightarrow{k_2} & E + P \\ & & + & & \\ & & I & & \\ & & \Big\updownarrow K_I^S & & \\ & & ESI & & \end{array} \tag{32}$$

$$\upsilon = \frac{\frac{\upsilon_{max}}{K_S} \cdot [S]}{1 + \frac{[S]}{K_S} + \frac{[S] \cdot [I]}{K_S \cdot K_I^S}} \tag{33}$$

$$\upsilon = \frac{\upsilon_{max} \cdot [S]}{K_S + [S] + \frac{[S] \cdot [I]}{K_I^S}} \tag{34}$$

Compared to Eq. (30) the rate equation for uncompetitive inhibitors includes the same term for the equilibrium of decomposition of the ESI complex into E, S and I, but no term for an equilibrium of an EI complex.

$$\upsilon = \frac{\upsilon_{max} \cdot [S]}{K_S + [S] \cdot \left(1 + \frac{[I]}{K_I^S}\right)} \quad \Rightarrow \quad \upsilon = \frac{\frac{\upsilon_{max}}{\left(1 + \frac{[I]}{K_I^S}\right)} \cdot [S]}{\frac{K_S}{\left(1 + \frac{[I]}{K_I^S}\right)} \cdot [S]} \tag{35}$$

One special form of uncompetitive inhibition is *substrate inhibition*. Here a second substrate molecule binds at the ES complex resulting in an inactive ESS complex. This form of inhibition is often found and will be discussed below (see acylase kinetics, Fig. 7-20 A).

7.4.2.5

Reversibility of One-Substrate Reactions

Because of the *"principle of microscopic reversibility"* each molecular process (in contrast to a macroscopic process) may occur in both forward and backward directions. As a consequence the end product P of an enzymatic conversion can act as a competitive inhibitor of the enzyme or, depending on the thermodynamic equilibrium, be transformed to the substrate S. If the interconversion of the ES to the EP complex is the rate-determining step the rapid equilibrium assumption is valid and the rate equation can be derived easily.

$$E + S \overset{K_S}{\rightleftharpoons} ES \underset{k_B}{\overset{k_F}{\rightleftharpoons}} EP \overset{K_P}{\rightleftharpoons} E + P \tag{36}$$

$$\upsilon_P = \frac{\frac{\upsilon_{F,max}}{K_S} \cdot [S] - \frac{\upsilon_{B,max}}{K_P} \cdot [P]}{1 + \frac{[S]}{K_S} + \frac{[P]}{K_P}} \tag{37}$$

υ_P	(mmol L^{-1} min^{-1})	Reaction rate for the formation of the product P
$\upsilon_{F,max}$	(mmol L^{-1} min^{-1})	Maximum reaction rate of the forward reaction
$\upsilon_{B,max}$	(mmol L^{-1} min^{-1})	Maximum reaction rate of the backward reaction

Again the denominator represents the dissociation equilibria of the ES complex and the EP complexes. Both partial reactions, the forward and the backward reactions, are catalyzed simultaneously; both "substrates" S and P are competing for the same enzyme. If the $\upsilon_{B,max}$ value in Eq. (37) equals zero, the equation reflects competitive inhibition of the enzyme by the product P (see Eq.(27)).

Eq. (37) is a result of the rate equation of the forward reaction reduced by the rate equation of the backward reaction, both having the same denominator. The numerator represents the first-order kinetics of the forward and the backward reactions. If the equilibrium of the reaction is reached, the numerator becomes zero. From the equilibrium condition (Eq. (38)),

$$\frac{\upsilon_{F,max}}{K_S} \cdot [S] - \frac{\upsilon_{B,max}}{K_P} \cdot [P] = 0 \tag{38}$$

a correlation between the thermodynamic parameter *"equilibrium constant"* and the kinetic parameters can be derived (Eq. (39)):

$$K_{eq} = \frac{[P]_{eq}}{[S]_{eq}} = \frac{\upsilon_{F,max}}{K_S} : \frac{\upsilon_{B,max}}{K_P} \tag{39}$$

This relation, representing the ratio of the rate constants of the first order kinetics is known as the "*Haldane equation*". Haldane equations can be formulated for every kinetic model describing an equilibrium reaction, just by setting the numerator to zero.

An example may illustrate this principle:

Eq. (37) is the basic kinetic model for isomerizations and racemizations. For example, the kinetic constants of an alanine racemase of *Bacillus stearothermophilus* are found to be

$\upsilon_{\text{D-Ala}\rightarrow\text{L-Ala,max}} = 7.02\ \text{U mL}^{-1}$ $K_{\text{D-Ala}} = 3.42\ \text{mmol L}^{-1}$

$\upsilon_{\text{L-Ala}\rightarrow\text{D-Ala,max}} = 11.82\ \text{U mL}^{-1}$ $K_{\text{L-Ala}} = 5.76\ \text{mmol L}^{-1}$

The corresponding rate constants of the first order kinetics are

$\upsilon_{\text{D-Ala}\rightarrow\text{L-Ala,max}} / K_{\text{D-Ala}} = 2.052\ \text{min}^{-1}$

$\upsilon_{\text{L-Ala}\rightarrow\text{D-Ala,max}} / K_{\text{L-Ala}} = 2.052\ \text{min}^{-1}$

These rate constants are identical, resulting in $K_{eq} = 1$, a predictable result of racemization of D- or L-alanine.

This example shows that the rate constants of the first order kinetics determine the position of equilibrium of the reaction. In the case of reversible two-substrate reactions, the rate constants of the second order kinetics have to be used as shown below.

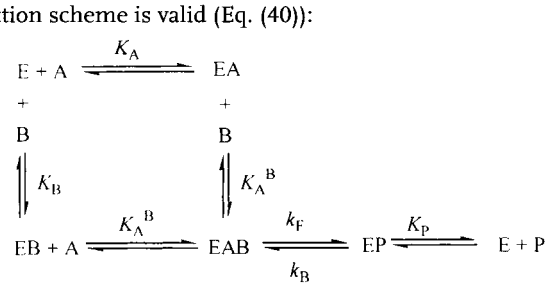
7.4.2.6
Two-Substrate Reactions

In most enzyme-catalyzed reactions two or more substrates are involved, such as in the enzyme-catalyzed aldol reaction, the cyanohydrin reaction or enzyme-catalyzed peptide synthesis (examples used before). For many reaction schemes kinetic models have been derived using the steady-state assumption. Some important reaction mechanisms and the corresponding rate equations are summarized in Table 7-1. An approach to the steady-state method and a detailed discussion of the resulting kinetic models is difficult and is not the aim of this chapter.

For a practicable approach, the "rapid equilibrium" assumption is applied and the structure of kinetic models of two substrate reactions is demonstrated for the case of a "*random bi-uni reaction*".

The binding of two substrates A and B to an enzyme may occur in a *compulsory order* or in a *random order*. If one product (uni) is formed out of two substrates (bi), the corresponding mechanisms are the "*ordered bi-uni*" mechanism and the "*random bi-uni mechanism*", respectively (water is not regarded as a substrate).

For a "*random bi-uni*" mechanism of an equilibrium reaction A + B ⇌ P the following reaction scheme is valid (Eq. (40)):



(40)

If the interconversion of the central EAB complex to the EP complex is rate determining and all other reactions are in a *"rapid equilibrium"*, the following rate equation for the random bi-uni reaction may be derived according to the above method:

$$\upsilon_P = \frac{\frac{\upsilon_{F,max}}{K_A \cdot K_B^A} \cdot [A] \cdot [B] - \frac{\upsilon_{B,max}}{K_P} \cdot [P]}{1 + \frac{A}{K_A} + \frac{B}{K_B} + \frac{P}{K_P} + \frac{A \cdot B}{K_A \cdot K_B^A}} \tag{41}$$

Again the denominator represents all enzyme-substrate equilibria, e.g. Eq. (42):

$$K_A \cdot K_B^A = \frac{[E] \cdot [A] \cdot [B]}{[EAB]} \quad => \quad \frac{[A] \cdot [B]}{K_A \cdot K_B^A} = \frac{[EAB]}{[E]} \tag{42}$$

The numerator represents the second-order kinetics of the forward reaction reduced by the first-order kinetics of the back reaction.

A Haldane equation can be formulated by setting the numerator to zero, resulting in a relation for the equilibrium constant (Eq. (43)):

$$K_{eq} = \frac{[A] \cdot [B]}{[P]} = \frac{\upsilon_{B,max} \cdot K_A \cdot K_B^A}{K_P \cdot \upsilon_{F,max}} \tag{43}$$

In the absence of the product P, Eq. (41) may be rearranged by multiplication of numerator and denominator by $K_A K_B{}^A$ (respectively $K_B K_A{}^B$, which is identical to $K_A K_B{}^A$, compare to Eq. (40)).
Then Eq. (44) results:

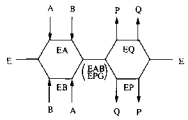
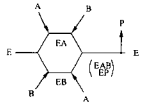
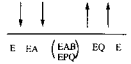
$$\upsilon_P = \frac{\upsilon_{F,max} \cdot [A] \cdot [B]}{K_A \cdot K_B^A + K_B^A \cdot [A] + K_A^B \cdot [B] + [A] \cdot [B]} \tag{44}$$

Only if a further identity $K_A = K_A{}^B$ is assumed can Eq. (44) be rearranged to Eq. (45):

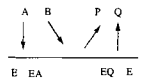
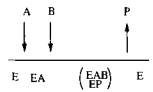
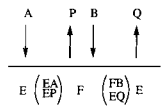
$$\upsilon_P = \frac{\upsilon_{F,max} \cdot [A]}{K_A + [A]} \cdot \frac{[B]}{K_B^A + [B]} \tag{45}$$

This rate equation is called "Michaelis-Menten double substrate kinetics". It is a formal multiplication of two Michaelis-Menten models for both substrates A and B. This model can be used to describe rate kinetics of two substrate reactions in the absence of the product(s). The kinetic measurements have to be performed by varying the concentration of one substrate keeping the concentration of the second substrate at a constant value well above the K_M value. The model cannot be used if back reactions occur and an equilibrium has to be described by an appropriate Haldane equation.

Table 7-1. Summary of different two substrate reaction mechanisms: reactions schemes and rate equations, according to the steady state assumption[45, 102].

rate equation / reaction scheme	numerator		kinetic constants
	bi – bi reaction	*bi – uni reaction*	total
	$\frac{\upsilon_f}{K_I^A \cdot K_M^B} \cdot A \cdot B - \frac{\upsilon_b}{*^1(K_M^P \cdot K_I^Q)} \cdot P \cdot Q$ $*^2(K_I^P \cdot K_M^Q)$	$\frac{\upsilon_f}{K_I^A \cdot K_M^B} \cdot A \cdot B - \frac{\upsilon_b}{K_M^P} \cdot P$	
Random bi bi $*^1$ or $*^2$ numerator $K_M^P \cdot K_I^Q = K_I^P \cdot K_M^Q$ 	denominator $1 + \frac{A}{K_I^A} + \frac{B}{K_I^B} + \frac{P}{K_I^P} + \frac{Q}{K_I^Q} + \frac{A \cdot B}{K_I^A \cdot K_M^B} + \frac{P \cdot Q}{K_M^P \cdot K_I^Q}$		$\upsilon_f \upsilon_b$ $A : K_I$ $B : K_I K_M$ $P : K_M$ $Q : K_I K_M$ $\Sigma 8$
Random bi uni $K_M^P = K_I^P$ 	$1 + \frac{A}{K_I^A} + \frac{B}{K_I^B} + \frac{P}{K_I^P} + \frac{A \cdot B}{K_I^A \cdot K_M^B}$		$\upsilon_f \upsilon_b$ $A : K_I$ $B : K_I K_M$ $P : K_I$ $\Sigma 6$
Ordered bi bi $*^1$ numerator 	$1 + \frac{A}{K_I^A} + \frac{B \cdot K_M^A}{K_M^B \cdot K_I^A} + \frac{P \cdot K_M^Q}{K_M^P \cdot K_I^Q} + \frac{Q}{K_I^Q} + \frac{A \cdot B}{K_I^A \cdot K_M^B} + \frac{P \cdot Q}{K_M^P \cdot K_I^Q} + \frac{A \cdot P \cdot K_M^Q}{K_I^A \cdot K_M^P \cdot K_I^Q} + \frac{B \cdot Q \cdot K_M^A}{K_M^B \cdot K_I^Q \cdot K_I^A} + \frac{A \cdot B \cdot P}{K_I^A \cdot K_M^B \cdot K_I^P} + \frac{B \cdot P \cdot Q}{K_I^B \cdot K_M^P \cdot K_I^Q}$		$\upsilon_f \upsilon_b$ $A : K_I$ $B : K_I K_M$ $P : K_M$ $Q : K_I K_M$ $\Sigma 10$

$*^{1,2}$ numerator according to reaction scheme

reaction scheme \ rate equation	numerator	kinetic constants
	bi – bi reaction: $\frac{\upsilon_f}{K_I^A \cdot K_M^B} \cdot A \cdot B - \frac{\upsilon_b}{*^1(K_M^P \cdot K_I^Q)} \cdot P \cdot Q$ *bi – uni reaction*: $\frac{\upsilon_f}{K_I^A \cdot K_M^B} \cdot A \cdot B - \frac{\upsilon_b}{K_M^P} \cdot P$ $*^2(K_I^P \cdot K_M^Q)$	total
Theorell-Chance *[1] numerator 	$1 + \frac{A}{K_I^A} + \frac{B \cdot K_M^A}{K_M^B \cdot K_I^A} + \frac{P \cdot K_M^Q}{K_M^P \cdot K_I^Q} + \frac{Q}{K_I^Q} + \frac{A \cdot B}{K_I^A \cdot K_M^B} + \frac{P \cdot Q}{K_M^P \cdot K_I^Q} + \frac{A \cdot P \cdot K_M^Q}{K_I^A \cdot K_M^P \cdot K_I^Q} + \frac{B \cdot Q \cdot K_M^A}{K_M^B \cdot K_I^Q \cdot K_I^A}$	$\upsilon_f \upsilon_b$ $A: K_I K_M$ $B: K_M$ $P: K_M$ $Q: K_I K_M$ $\Sigma 8$
Ordered bi uni 	$1 + \frac{A}{K_I^A} + \frac{B \cdot K_M^A}{K_M^B \cdot K_I^A} + \frac{P}{K_M^P} + \frac{A \cdot B}{K_I^A \cdot K_M^B} + \frac{B \cdot P}{K_I^B \cdot K_M^P}$	$\upsilon_f \upsilon_b$ $A: K_I K_M$ $B: K_I K_M$ $P: K_M$ $\Sigma 7$
Ping Pong bi bi *[2] numerator 	$\frac{A}{K_I^A} + \frac{B \cdot K_M^A}{K_M^B \cdot K_I^A} + \frac{P}{K_I^P} + \frac{Q \cdot K_M^P}{K_M^Q \cdot K_I^P} + \frac{A \cdot B}{K_I^A \cdot K_M^B} + \frac{P \cdot Q}{K_I^P \cdot K_M^Q} + \frac{A \cdot P}{K_I^A \cdot K_I^P} + \frac{B \cdot Q \cdot K_M^A}{K_M^B \cdot K_I^Q \cdot K_I^A}$	$\upsilon_f \upsilon_b$ $A: K_I K_M$ $B: K_M$ $P: K_I K_M$ $Q: K_I K_M$ $\Sigma 9$

*[1, 2] numerator according to reaction scheme

Footnote to Table 7-1.
The above reaction schemes are described according to the notion of Cleland[116]. The substrates binding to the enzyme are indicated as A and B according to the order of binding; the products are indicated as P and Q, respectively.
The different mechanisms may be explained in short as follows (for a detailed discussion the reader is referred to textbooks, listed above).

Random bi bi:	Both substrates A and B may bind; also the products P and Q may dissociate from the enzyme in a random order. (Example: *E. coli* galactokinase)
Random bi uni:	Two substrates A and B and only one product P are involved (water is not considered). (Example: aminoacylase, see above)
Ordered bi bi:	The substrates and products bind to or dissociate from the enzyme in a specific order. (Example: in most dehydrogenase-catalyzed reactions the coenzyme has to bind first; hexokinase)
Theorell-Chance:	This special case of the ordered bi-bi mechanism occurs if the first product P dissociates from the enzyme very rapidly and an EAB-, EPQ-complex does not occur in a significant concentration. (Example: alcohol-dehydrogenase)
Ordered bi uni:	Two substrates and only one product are involved. (Example: Neu5Ac-aldolase and other aldolases)
Ping Pong bi bi:	In this mechanism a product P is released between the addition of two substrates. This mechanism is often found in group transfer reactions. (Example: transaminases, yeast transaldolases)

Above-mentioned and additional examples are given in[117,118]. Looking at the rate equations for the different mechanisms the following points can be identified:

- Corresponding to the above discussion about enzyme kinetics, the numerator is nearly identical for all different bi-bi-mechanisms (for bi-uni mechanisms, respectively), as the numerator characterizes the thermodynamic equilibrium of the reaction (which is independent of a kinetic mechanism).
- The denominator consists of terms characterizing all enzyme substrate equilibria.
- Depending on the mechanism every substrate A, B, P and Q requires one or two kinetic constants (designated as K_I and K_M) in order to describe its reciprocal action with the enzyme. According to the steady -state derivation of these rate equations, K_I and K_M are no longer simple dissociation constants (compare discussion about K_S and K_M). In some cases K_I is identical to a dissociation constant as described before, but most often these steady state parameters are defined by three and more rate constants. A verbal distinction between an inhibition constant; a Michaelis constant and a dissociation constant does not have a corresponding mechanistic scenario in all cases.
- The random mechanism in both cases is the simplest mechanism requiring only 8 (random bi-bi) and 6 (random bi-uni) kinetic constants. This mechanism should be tried first to fit data of a two-substrate reaction.
- The denominator may be supplemented by additional terms e.g. for inhibitory substances, not being described by basic mechanism (compare to discussion of the acylase kinetics).
- A distinction between the different mechanisms is best done using initial rate kinetic measurements as described in detail in the literature. For reaction engineering purposes only a proper fit of reactor data is desired, using a minimum amount of kinetic parameters for statistical reasons.

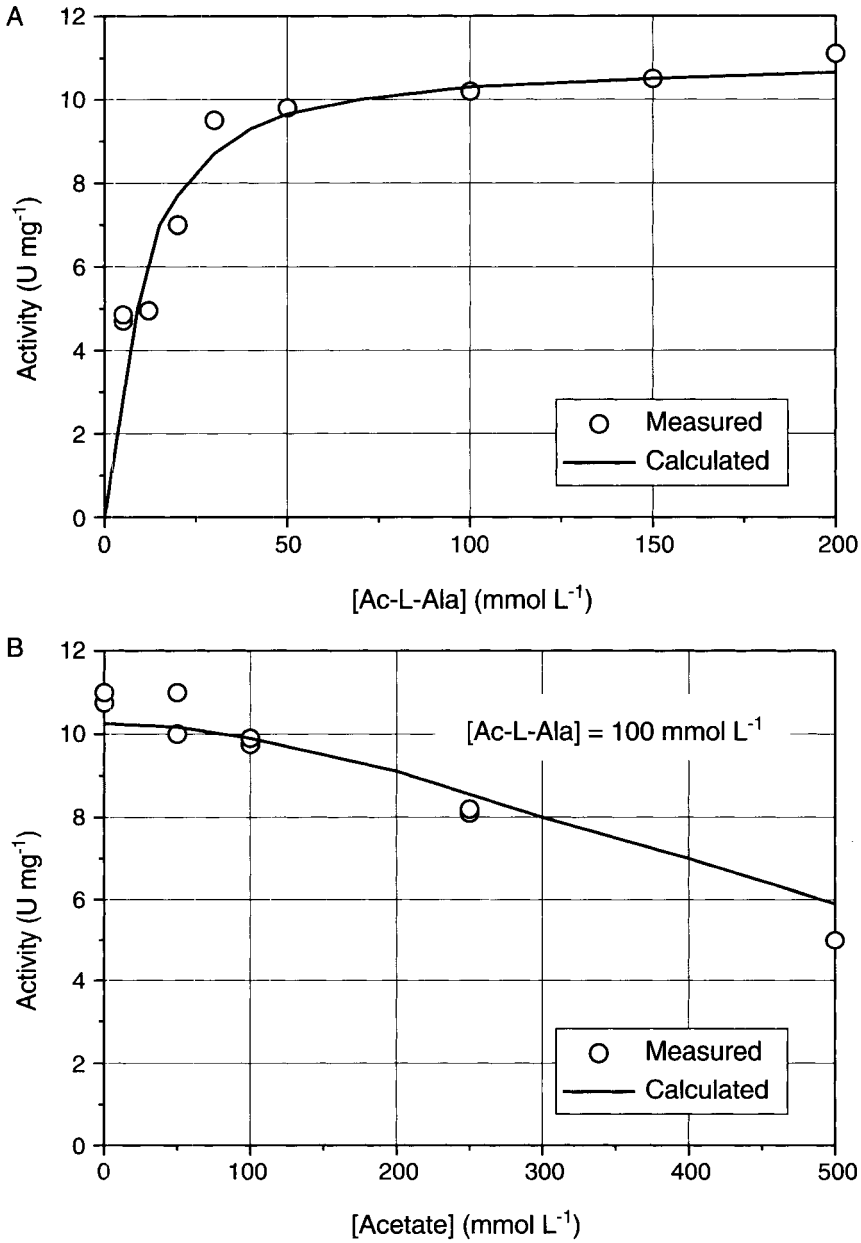
If the concentrations of the products P and Q in the models summarized in Table 7-1 are set to zero, the resulting rate equations will be identical to Eq. (44) in all cases with the exception of the "ping-pong" mechanism. This means that the Michaelis-Menten double substrate kinetics is valid under the above circumstances.

An example of the use of this model will be shown for a two enzyme system (see Sect. 7.4.3).

7.4.2.7

Kinetics of Aminoacylase as Example of a Random Uni-Bi Mechanism

Aminoacylase kinetics may be used as an example for demonstrating the measurement and modeling of enzyme kinetics. This reaction is of industrial importance in



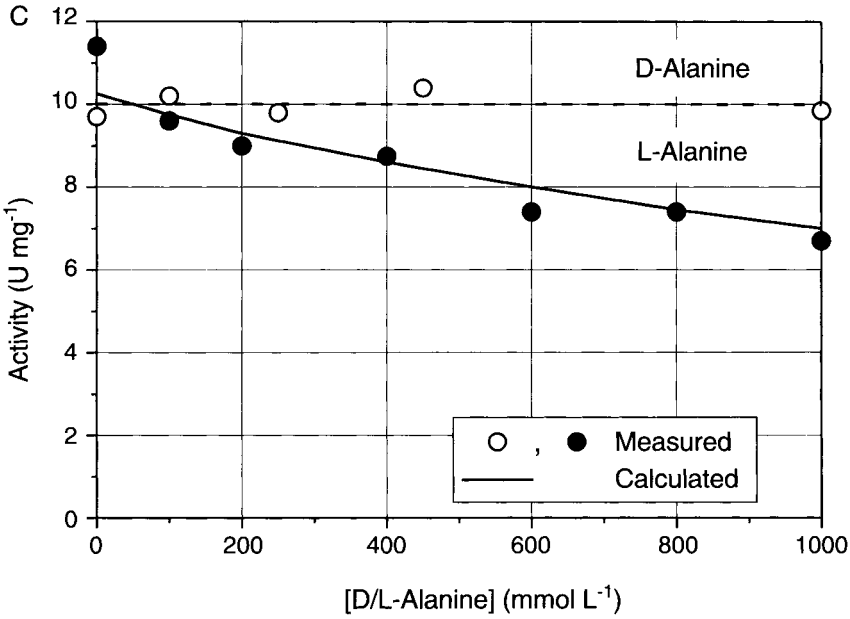


Figure 7-19. Kinetics of aminoacylase: effects on the activity of hydrolysis of acetyl-L-alanine[115] (initial rate measurements).

the production of L-amino acids by hydrolysis of acetyl-L-aminoacids. Since the maximum conversion is limited by the equilibrium of the reaction (compare with Fig. 7-6), kinetic measurements of both hydrolysis and synthesis have been performed and fitted by a kinetic model, using acetyl-L-alanine as an example. Additionally the influence of D-alanine was measured.

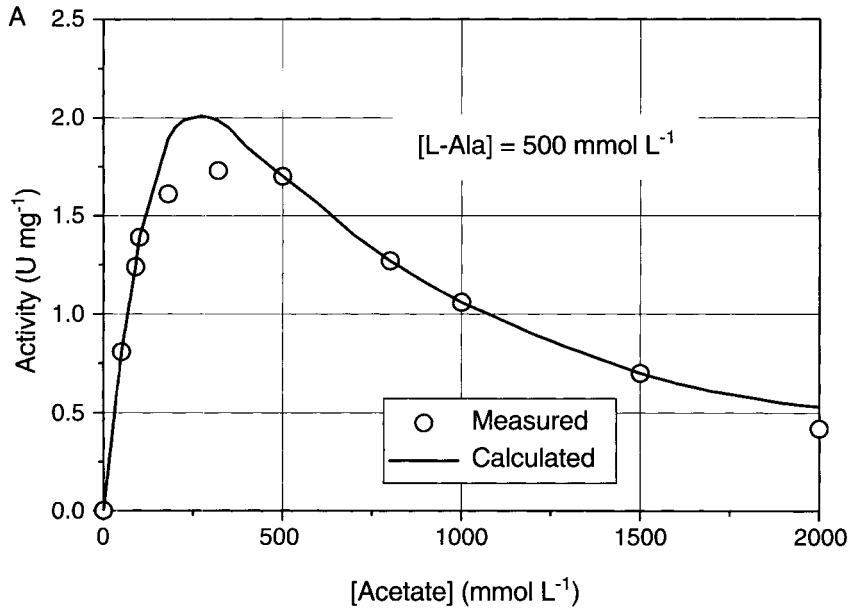
Fig. 7-19 shows influences of acetyl-L-alanine, acetate, D- and L-alanine on the hydrolysis activity of aminoacylase.

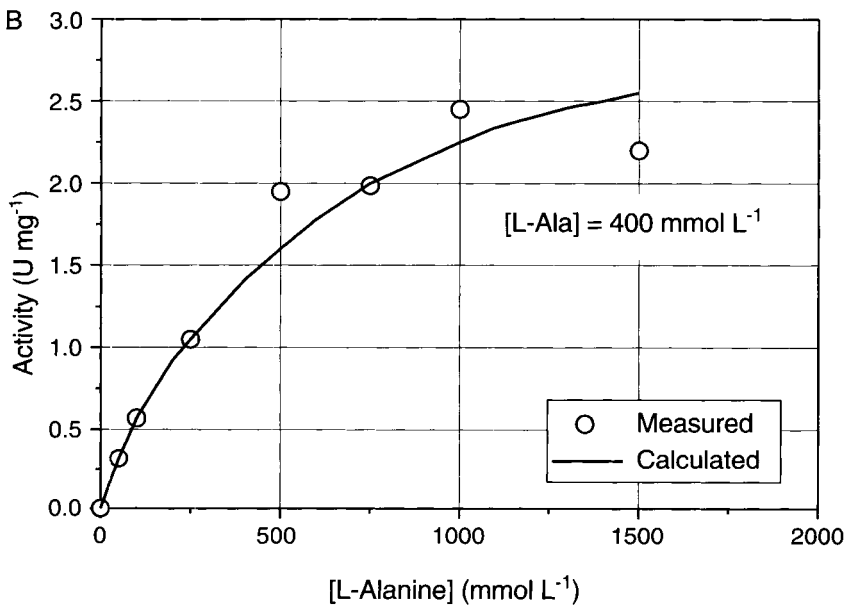
With respect to acetyl-L-alanine, the substrate of the hydrolysis reaction, the enzyme exhibits Michaelis-Menten kinetics (Fig. 7-19 A). The hydrolysis activity is inhibited by acetate (Fig. 7-19 B) and L-alanine (Fig. 7-19 C). These are the substrates of the synthesis reaction (see below) acting as inhibitory products.

Figure 7-20 shows the influences of acetate, L-alanine, D-alanine and acetyl-L-alanine on the specific activity of aminoacylase synthesizing acetyl-L-alanine.

With respect to acetate as the substrate (Fig. 7-20 A) the enzyme exhibits substrate inhibition (compare Eq. (33). Concerning the second substrate, L-alanine, Michaelis-Menten kinetics are apparent (Fig. 7-20 B). D-Alanine slightly inhibits the enzyme (Fig. 7-20 C).

On adding acetyl-L-alanine (as the product of the synthesis reaction) to a reaction mixture as specified in Fig. 7-20 D, the measured enzyme activity rapidly decreases, indicating product inhibition. Finally the measured activity reaches negative values. A negative activity here means a negative rate of acetyl-L-alanine synthesis, that is, hydrolysis.





This cannot be described by product inhibition alone and means the hydrolysis reaction commences above a concentration of 16.5 mmol L^{-1} acetyl-L-alanine. At this value the reaction is in its equilibrium position and the *measurable* enzyme activity

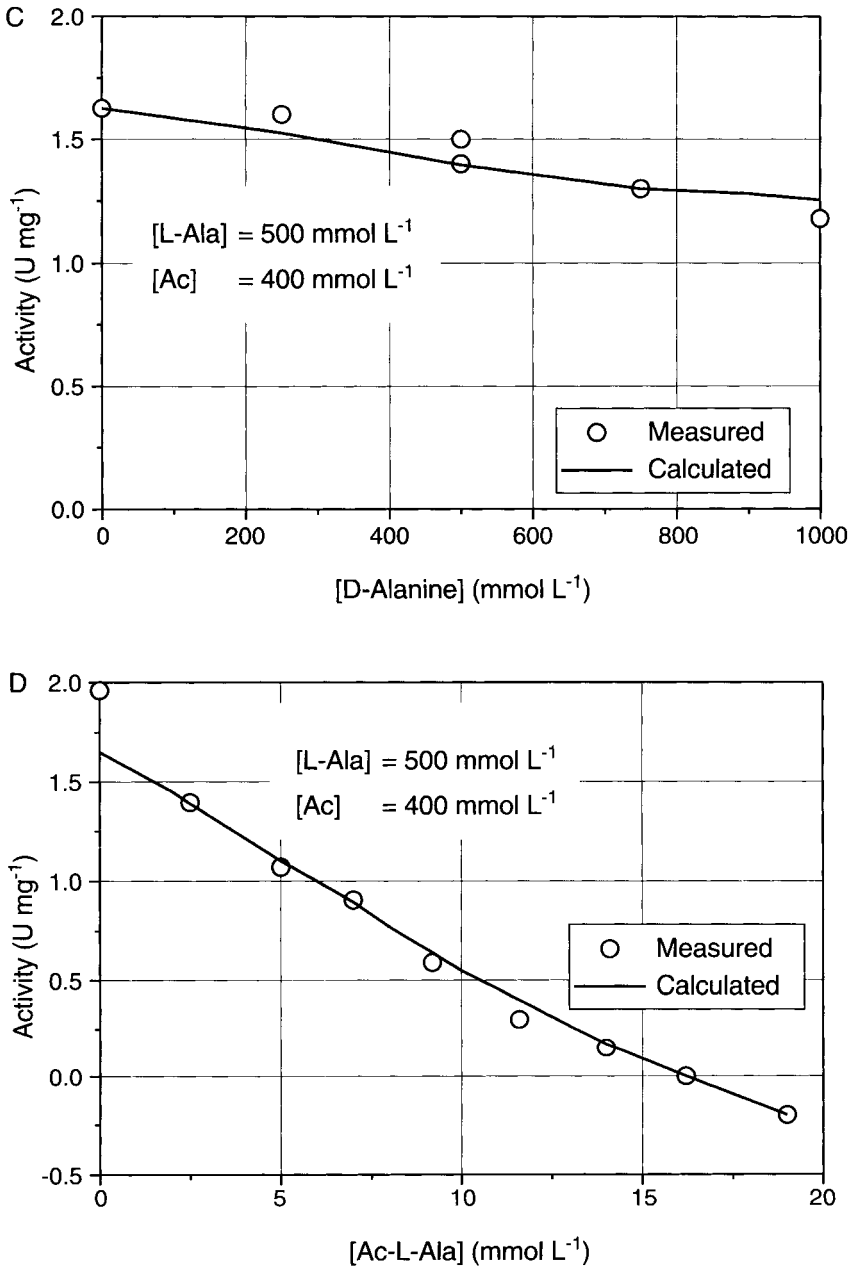
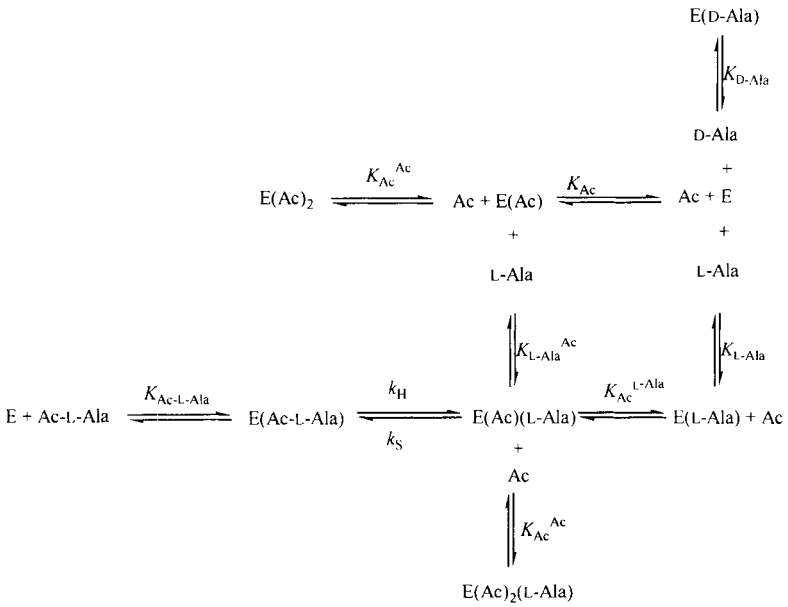


Figure 7-20. Kinetics of aminoacylase: effects on the activity of synthesis of acetyl-L-alanine (initial rate measurements) [115].

equals zero. Of course, the enzyme is not inactivated but there is no macroscopic turnover of substances.

Fig. 7-19 and Fig. 7-20 show a number of effects on the activity of aminoacylase, which cannot be described by a standard kinetic model alone. To derive a kinetic model for the aminoacylase and for describing all influences, a reaction scheme based on the rapid equilibrium random uni-bi model was used (compare with Eq. (40)). This model was supplemented, according to the above measured influences of substrate inhibition by acetate and inhibition by D-alanine. Consequently, the reaction scheme Eq. (40) had to be extended to yield equation (46) and the corresponding rate equation (47), which is modified by the additional equilibria:



(46)

The substrate inhibition of Fig. 7-20 A is represented by the complexes $E(Ac)_2$ and $E(Ac)_2$(L-Ala). This means that binding of acetate to all enzyme species besides the E(Ac-L-Ala) complex and the E(D-Ala)-complex is assumed. The rate equation of the random bi-uni model (Eq. (41)) is enlarged by three terms in the denominator according to the additional enzyme substrate complexes E(D-Ala), $E(Ac)_2$ and $E(Ac)_2$(L-Ala), whereas the numerator has the same structure as in Eq. (41).

The rate equation corresponding to the above reaction scheme can be written as follows:

Kinetic model of aminoacylase

$$-\frac{d[\text{Ac-L-Ala}]}{dt} = \frac{[E]_0 \cdot \frac{A_{H,max}}{K_{Ac\text{-}L\text{-}Ala}} \cdot [\text{Ac-L-Ala}] - [E]_0 \frac{A_{S,max}}{K_{Ac} \cdot K_{L\text{-}Ala}^{Ac}} \cdot [\text{Ac}] \cdot [\text{L-Ala}]}{1 + \frac{[\text{Ac}]}{K_{Ac}} + \frac{[\text{D-Ala}]}{K_{D\text{-}Ala}} + \frac{[\text{L-Ala}]}{K_{L\text{-}Ala}} + \frac{[\text{Ac-L-Ala}]}{K_{Ac\text{-}L\text{-}Ala}} + \frac{[\text{Ac}] \cdot [\text{L-Ala}]}{K_{Ac} \cdot K_{L\text{-}Ala}^{Ac}} + \frac{[\text{Ac}]^2}{K_{Ac} \cdot K_{Ac}^{Ac}} + \frac{[\text{Ac}]^2 \cdot [\text{L-Ala}]}{K_{Ac} \cdot K_{L\text{-}Ala}^{Ac} \cdot K_{Ac}^{Ac}}} \quad (47)$$

$A_{S,max}$	(U mg^{-1})	Maximum activity of the synthesis of Ac-L-Ala
$A_{H,max}$	(U mg^{-1})	Maximum activity of the hydrolysis of Ac-L-Ala
		Dissociation constant of the EX complex with
K_{Ac}	(mmol L^{-1})	X = acetate
$K_{L\text{-}Ala}$	(mmol L^{-1})	X = L-alanine
$K_{D\text{-}Ala}$	(mmol L^{-1})	X = D-alanine
$K_{Ac\text{-}L\text{-}Ala}$	(mmol L^{-1})	X = acetyl-L-alanine
$K^{Ac}{}_{Ac}$	(mmol L^{-1})	dissociation constant of the E(Ac)$_2$-complex with release of acetate
$K^{Ac}{}_{L\text{-}Ala}$	(mmol L^{-1})	dissociation constant of the E(Ac)(l-Ala)complex with release of L-alanine

This kinetic model contains 8 parameters: one parameter for each of the four components D,L-alanine, acetate and acetyl-L-alanine, one parameter ($K_{L\text{-}Ala}{}^{Ac}$) for the second substrate in the bimolecular synthesis reaction, one parameter describing the substrate inhibition and two parameters describing the hydrolysis and the synthesis rate. This is the minimum number of parameters necessary to describe the above influences on enzyme activity (*cf.* Table 7-1).

Eq. (47) was used for a simultaneous fit of all kinetic data measured under initial rate conditions (Figs. 7-19 and 7-20). Separate fitting of each curve gives a better coincidence in every single case, but the optimized kinetic parameter will vary from fit to fit.

The optimized kinetic parameters are summarized in Table 7-2.

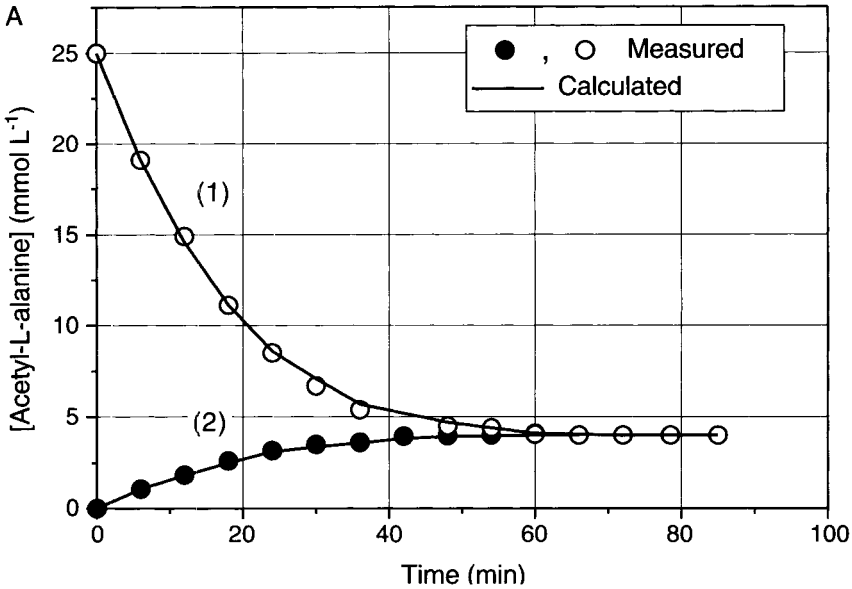
As described above, the rate equation can be set to zero yielding the equilibrium condition of the reaction (Haldane equation). Using the kinetic data, K_{eq} is calculated to be 12.2 mol L^{-1}.

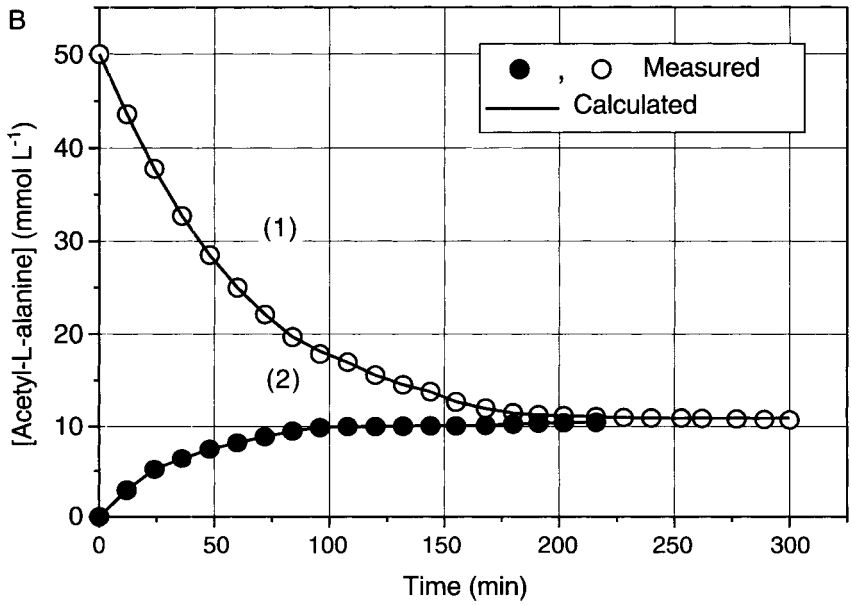
$$K_{eq} = \frac{[\mathrm{Ac}]\cdot[\mathrm{L-Ala}]}{[\mathrm{Ac-L-Ala}]} = \frac{A_{\mathrm{H,max}} \cdot K_{\mathrm{Ac}} \cdot K^{\mathrm{Ac}}_{\mathrm{L-Ala}}}{A_{\mathrm{S,max}} \cdot K_{\mathrm{Ac-L-Ala}}} \tag{48}$$

As ultimate proof of the kinetic model, a fit of time-courses of batch reactor experiments was performed (Fig. 7-21). Initial concentrations of the components over a significant range were chosen to yield hydrolysis conditions (1) and synthesis conditions (2) respectively. Additionally, the equilibrium positions of corresponding experiments A, B and C were chosen to be identical. Figure 7-21 shows a good correlation of calculated and measured data over the whole range of the conversion, for hydrolysis as well as for synthesis.

Table 7-2. Kinetic parameters of aminoacylase.

Hydrolysis of Ac-L-Ala			Synthesis of Ac-L-Ala		
$A_{H,max}$	11.2	U mg^{-1}	$A_{S,max}$	118.1	U mg^{-1}
$K_{Ac\text{-}L\text{-}Ala}$	8.9	mmol L^{-1}	K_{Aca}	1070	mmol L^{-1}
			$K_{Ac}{}^{Ac}$	26.9	mmol L^{-1}
			$K_{L\text{-}Ala}{}^{Ac}$	1070	mmol L^{-1}
			$K_{L\text{-}Ala}$	177	mmol L^{-1}
			$K_{D\text{-}Ala}$	270	mmol L^{-1}


A
Measured
Calculated
(1)
(2)
[Acetyl-L-alanine] (mmol L-1)
Time (min)
0
5
10
15
20
25
0
20
40
60
80
100


B
Measured
Calculated
(1)
(2)
[Acetyl-L-alanine] (mmol L-1)
Time (min)
0
10
20
30
40
50
0
50
100
150
200
250
300

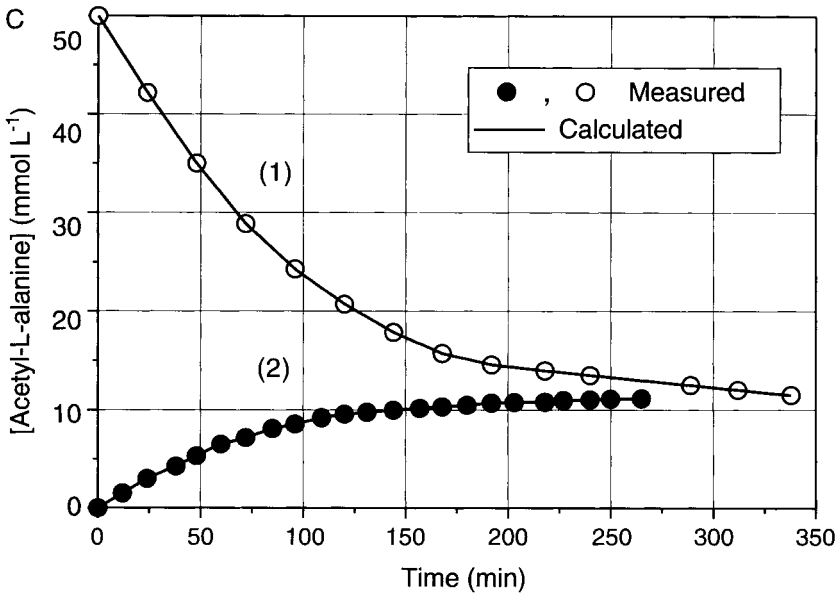


Figure 7-21. Time-course data of hydrolysis and synthesis of acetyl-L-alanine using initial conditions as specified in Table 7–3 [115].

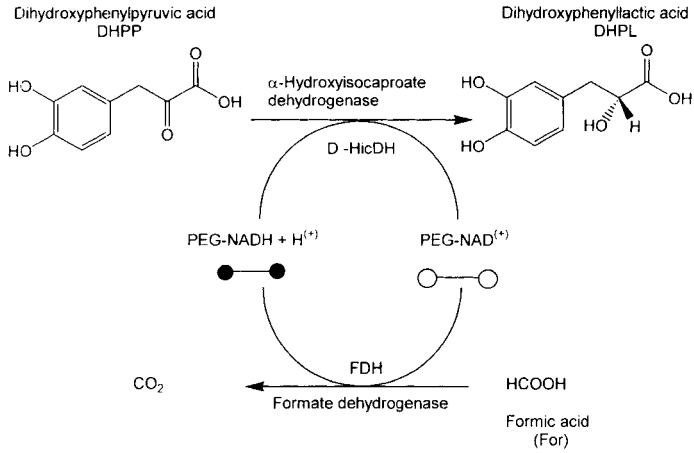
Table 7-3. Starting conditions for the experiments shown in Fig. 7-21.

Concentrations		A(1)	A(2)	B(1)	B(2)	C(1)	C(2)
$[Ac]_0$	(mmol L^{-1})	175	200	150	200	300	400
$[D,L\text{-}Ala]_0$	(mmol L^{-1})	450	500	1400	1500	1300	1500
$[Ac\text{-}L\text{-}Ala]_0$	(mmol L^{-1})	25	0	50	0	100	0
$\Sigma[L\text{-}Ala]_0$	(mmol L^{-1})	200	200	200	200	400	400
$\Sigma[L\text{-}Ala]_0$	(mmol L^{-1})	250	250	750	750	750	750

7.4.3
Kinetics of Multiple Enzyme Systems

If two or more enzymes are involved in coupled reactions, the influence of all substances present in the reaction mixture on the activity of all enzymes has to be studied and considered in the kinetic model. Knowing the kinetic behavior of the single enzymes, coupling can be done by writing mass balances.

As an example, the reduction of dihydroxyphenylpyruvic acid (DHPP) to dihydroxyphenyllacetic acid (DHPL), a precursor of rosmarinic acid, is presented in Eq. (49) [119].



(49)

The reaction is catalyzed by D-hydroxyisocaproate dehydrogenase (D-HicDH). The essential cofactor PEG-NADH is regenerated from PEG-NAD$^+$ by a second enzyme, formate dehydrogenase (FDH). By coupling to water-soluble polyethyleneglycol with a molar mass of 20 000 g mol^{-1}, the cofactor can be retained, together with the enzymes, by an ultrafiltration membrane, and the whole process may be performed continuously in an enzyme membrane reactor.

The kinetic models of D-HicDH and FDH are described as follows (Eqs. (50) and (51)):

Kinetic model of D-HicDH

$$\upsilon_1 = \frac{d[\mathrm{DHPL}]}{dt} = [\mathrm{D-HicDH}]_0 \cdot A_{\mathrm{D-HicKH,max}} \cdot \frac{[\mathrm{DHPP}]}{K_{\mathrm{KHPP}} + [\mathrm{DHPP}]} \cdot \frac{[\mathrm{PEG-NADH+H^+}]}{K_{\mathrm{PEG-NADH+H^+}} \cdot \left(1 + \frac{[\mathrm{PEG-NAD^+}]}{K_{\mathrm{PEG-NAD^+}}}\right) + [\mathrm{PEG-NADH+H^+}]} \tag{50}$$

Kinetic model of FDH

$$\upsilon_2 = -\frac{d[\mathrm{For}]}{dt} = [\mathrm{FDH}]_0 \cdot A_{\mathrm{FDH,max}} \cdot \frac{[\mathrm{For}]}{K_{\mathrm{For}} + [\mathrm{For}]} \cdot \frac{[\mathrm{PEG-NAD^+}]}{K_{\mathrm{PEG-NAD^+}} \cdot \left(1 + \frac{[\mathrm{PEG-NADH+H^+}]}{K_{\mathrm{PEG-NADH+H^+}}}\right) + [\mathrm{PEG-NAD^+}]} \tag{51}$$

In this case Michaelis-Menten double substrate kinetics is chosen (compare to Eq. (45)). The system involving D-HicDH with DHPP and PEG-NADH as substrates exhibits Michaelis-Menten kinetics for both substrates and competitive product inhibition by PEG-NAD$^+$. FDH also shows Michaelis-Menten kinetics for both substrates formate and PEG-NAD$^+$ and competitive product inhibition by PEG-

NADH. Both inhibiting effects are taken into consideration by an additional term in the denominator. This formal procedure is valid as it allows a correct fit of all initial rate data (not shown).

For a batch reaction (compare to Sect. 7.5) mass balances of all components of this system may be formulated as follows (Eqs. (52–54)):

$$\frac{d[DHPL]}{dt} = -\frac{d[DHPP]}{dt} = \upsilon_1 \tag{52}$$

$$-\frac{d[For]}{dt} = \upsilon_2 \tag{53}$$

$$\frac{d[PEG-NADH+H^+]}{dt} = -\frac{d[PEG-NAD^+]}{dt} = \upsilon_2 - \upsilon_1 \tag{54}$$

The two enzymatic reactions are coupled by PEG-NADH and PEG-NAD$^+$. This stoichiometric coupling does not affect enzyme kinetics but has to be considered when writing the mass balances. The discussion of this system will be continued in Sect. 7.5 where some implications of coupled enzyme systems on reactor design are described.

7.5 Enzyme Reactors

7.5.1 Basic Reaction Engineering Aspects

First, some basic terms of chemical reaction engineering will be discussed before introducing the field of enzyme reactors. For further reading, textbooks are available[120–126].

The mode of reactor operation can be classified as "batchwise" or "continuous".

Batch reactions are started by filling a reactor with the reaction mixture and stopped after reaching the desired conversion. A steady state is only reached at equilibrium conversion of the reaction. A typical batch reactor is represented by the stirred tank reactor.

Continuous reactions are characterized by a continuous substrate feed and product output. A residence time of the reaction mixture within the total reactor volume V can be defined by Eq. (55):

$$\tau = \frac{V}{F} \tag{55}$$

τ	(h)	Residence time
V	(L)	Total reactor volume
F	(L h^{-1})	Substrate feed rate

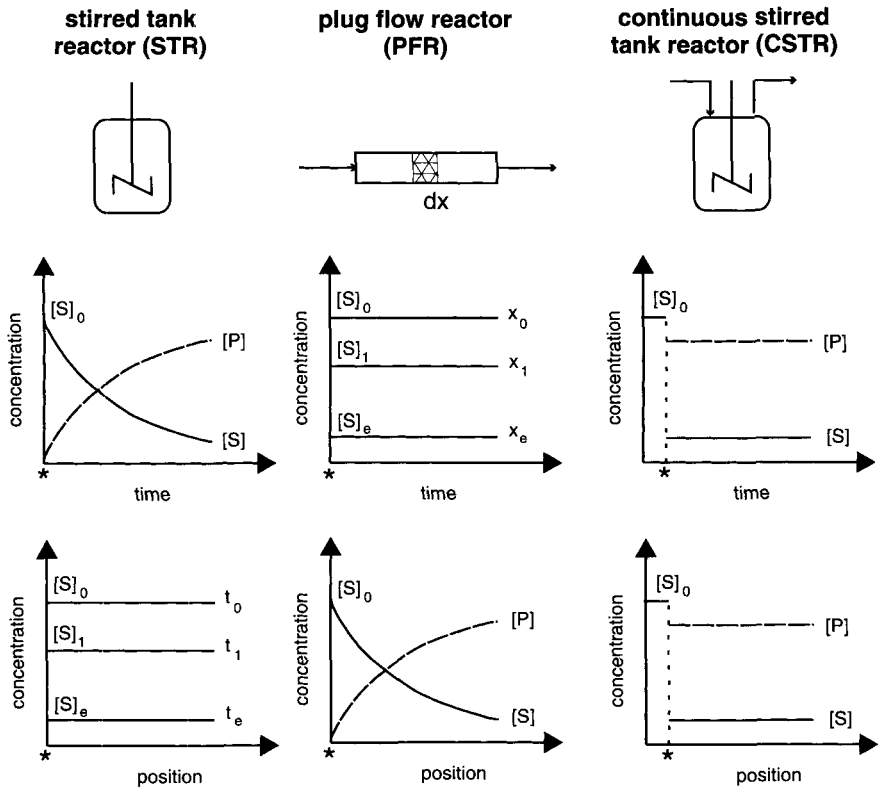


Figure 7-22. Comparison of stirred tank reactor, plug flow reactor and continuous stirred tank reactor (Reaction: S → P; asterisks indicate time or position of substrate entering the reactor).

After a certain time a steady state will be reached within the reactor, meaning that concentrations of substrates and products do not change. Typical continuous reactors are the plug flow reactor (PFR) and the continuous stirred tank reactor (CSTR). A comparison of these different reactor types is given in Fig. 7-22.

The differences between the reactors may be described by showing concentration profiles of substrate and product as a function of reactor position and time respectively.

- The stirred tank reactor (STR) in batch mode exhibits a decreasing substrate concentration and increasing product concentration with time, independently of the position within the reactor (the reactor is "well mixed", meaning that there are no gradients within the reactor).
- The plug flow reactor (PFR) presents the same concentration curve along the reactor length, which is shown for the tank reactor with reaction time. In the steady state, the concentrations of substrates and products at distinct positions of the reactor do not change with time. The reactor has plug flow characteristics,

implying flow of reaction mixture through the reactor in the form of a plug without any axial mixing.
- The continuous stirred tank reactor (CSTR) in the steady state exhibits constant concentrations as a function of both time and position. The exit stream from this reactor has the same composition as the fluid within the reactor; the CSTR is also a well-mixed reactor.

A very common variation of the CSTR is a cascade of n CSTRs. With an increasing number of reaction vessels, the cascade approximates to the plug-flow reactor. The product concentration increases stepwise from vessel to vessel. For example, a two-stage cascade can be used to overcome effects of product inhibition, e.g. in the synthesis of L-*tert*-leucine [42] or GDP-Man [144, 145].

The basis for calculating reactor operation conditions is the formulation of mass balances for all reaction components for the distinct reactor type. The mass balances for the above reactors can be formulated as follows:

Stirred tank reactor: as no fluid enters or leaves the reactor the mass balance of every compound is defined by the reaction rate only. To determine the time t necessary to reach the desired conversion x the reciprocal rate equation has to be integrated from zero to the desired conversion x:

With the definition of conversion shown in Eq. (56),

$$x = \frac{[S]_0 - [S]}{[S]_0} \Rightarrow \frac{dx}{d[S]} = -\frac{1}{[S]_0} \tag{56}$$

the equation for the reaction time t can be derived from the reaction rate v (Eq. (57)):

$$\upsilon = -\frac{d[S]}{dt} \Rightarrow dt = -\frac{d[S]}{\upsilon} = \frac{[S]_0 \cdot dx}{\upsilon} \Rightarrow t = [S]_0 \cdot \int_0^x 1/\upsilon \cdot dx \tag{57}$$

Plug flow reactor: The change of reaction rate within a unit volume passing through the length of the reactor is equivalent to a change corresponding to the residence time τ within the reactor. To determine the residence time τ necessary to reach the desired conversion x, it has to be integrated again over the whole range of conversion. Eq. (57) can also be used for the PFR simply by replacing t by τ (Eq. (58)).

$$\tau = [S]_0 \cdot \int_0^x 1/\upsilon \cdot dx \tag{58}$$

Continuous stirred tank reactor: In the case of CSTR, the change of concentration of a substrate S within the reactor ("accumulation") is brought about by two terms:

- The "convection term", which describes the change of concentration of S within the reactor as an effect of the influx of the substrate into the reactor, reduced by the efflux out of the reactor.
- The "reaction term", which describes the change of concentration of S as result of the reaction S → P.

accumulation = convection + reaction

$$\frac{d[S]}{dt} = \frac{[S]_0 - [S]}{\tau} - \upsilon \tag{59}$$

At steady state, the concentrations within the reactor will not change, meaning that the accumulation term equals zero. By using the definition of x (Eq. (5)), the residence time τ necessary to reach the desired conversion x can be calculated (Eq. (60)):

$$0 = \frac{[S]_0 - [S]}{\tau} - \upsilon \Rightarrow \tau = \frac{[S]_0 - [S]}{\upsilon} \Rightarrow \tau = [S]_0 \cdot \frac{1}{\upsilon} \cdot x \tag{60}$$

τ is given by a simple multiplication of x by the reciprocal reaction rate at this steady state conversion x. The situation can be further illustrated by the following plot (Fig. 7-23):

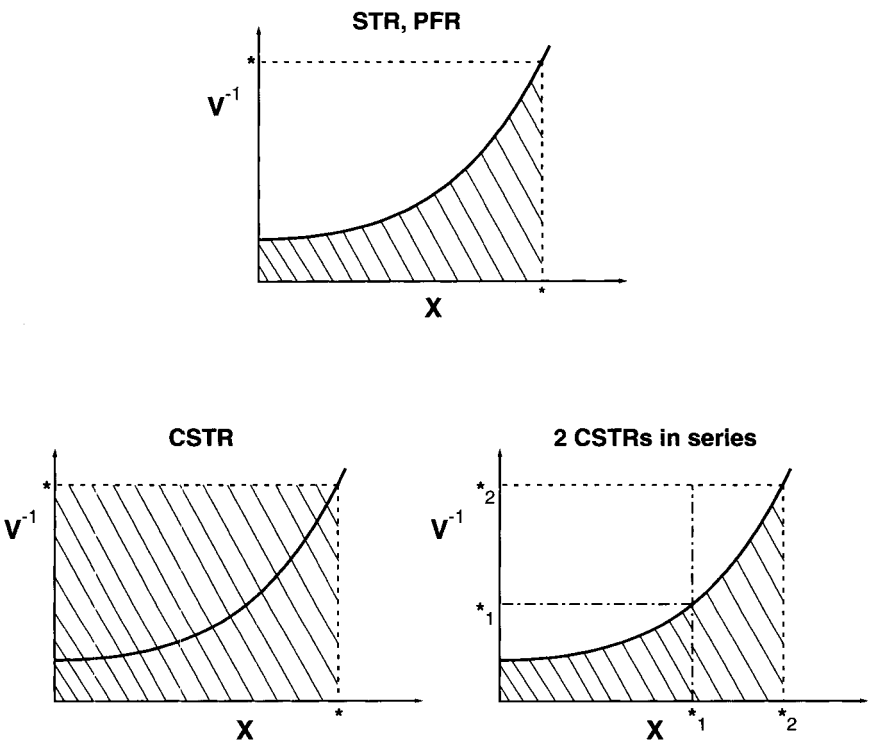


Figure 7-23. Reactor design from $1/\nu = f(x)$ plots (shadowed area represents $\tau[S]_0^{-1}$; asterisks represent reactor outlet conditions).

If the reciprocal reaction rate is plotted as a function of conversion the shadowed areas represent the residence time necessary to reach a given conversion x (Eqs. (61) and (62)).

$$\frac{\tau}{[S]_0} = \int_0^x 1/\upsilon \cdot dx \qquad \text{(for stirred tank and plug flow reactor)} \tag{61}$$

$$\frac{\tau}{[S]_0} = 1/\upsilon \cdot dx \qquad \text{(for continuous stirred tank reactor)} \tag{62}$$

Obviously the residence time is higher in the case of the CSTR compared to the STR and the PFR if the reaction rate continuously decreases with conversion, this most often being the case in enzymatic reactions.

Minimizing the necessary τ value for reaching a defined conversion x (at constant $[S]_0$ value) can be done graphically using these $1/v = f(x)$ plots (Fig. 7-23). For different reactors, the area may be determined according to the above method and the appropriate reactor is given by the minimal area. For example the τ value of a CSTR can be minimized by using two CSTRs in series. The steady state reaction mixture of the first reactor (conversion x_1) is fed into the second reactor, yielding an overall conversion x_2.
With the relation

$$\upsilon = [E]_0 \cdot A \qquad \text{(compare to Eq. 23)} \tag{63}$$

Eq. (61) and Eq. (62) may be written as

$$\frac{[E]_0 \cdot \tau}{[S]_0} = \int_0^x 1/A \cdot dx \quad \text{(for stirred tank and plug flow reactor)} \tag{64}$$

$$\frac{[E]_0 \cdot \tau}{[S]_0} = 1/A \cdot x \qquad \text{(for continuous stirred tank reactor)} \tag{65}$$

This means that the conversion x of a reaction at a constant $[S]_0$ value depends on the extent of activity during the conversion on the one hand and on the product $[E]_0 \cdot \tau$ on the other. Further, this means that lowering the enzyme concentration by a factor y and raising the residence time by the same factor y gives the same conversion x. This is the so-called "$[E]_0 \cdot \tau$-concept", valid for all three reactors.

Up to now general reaction engineering principles have been discussed. Now we turn to enzyme reactor design.

The suitability of different reactors is demonstrated for two typical enzyme kinetic examples, involving substrate inhibition in one case and product inhibition in the other (Fig. 7-24).
The kinetics within Fig. 7-24 do not represent initial rate kinetics, but the reaction rate during the conversion $S \rightarrow P$ plotted versus the remaining substrate concentration (initial concentration $[S]_0 = 1$ mmol L^{-1}).

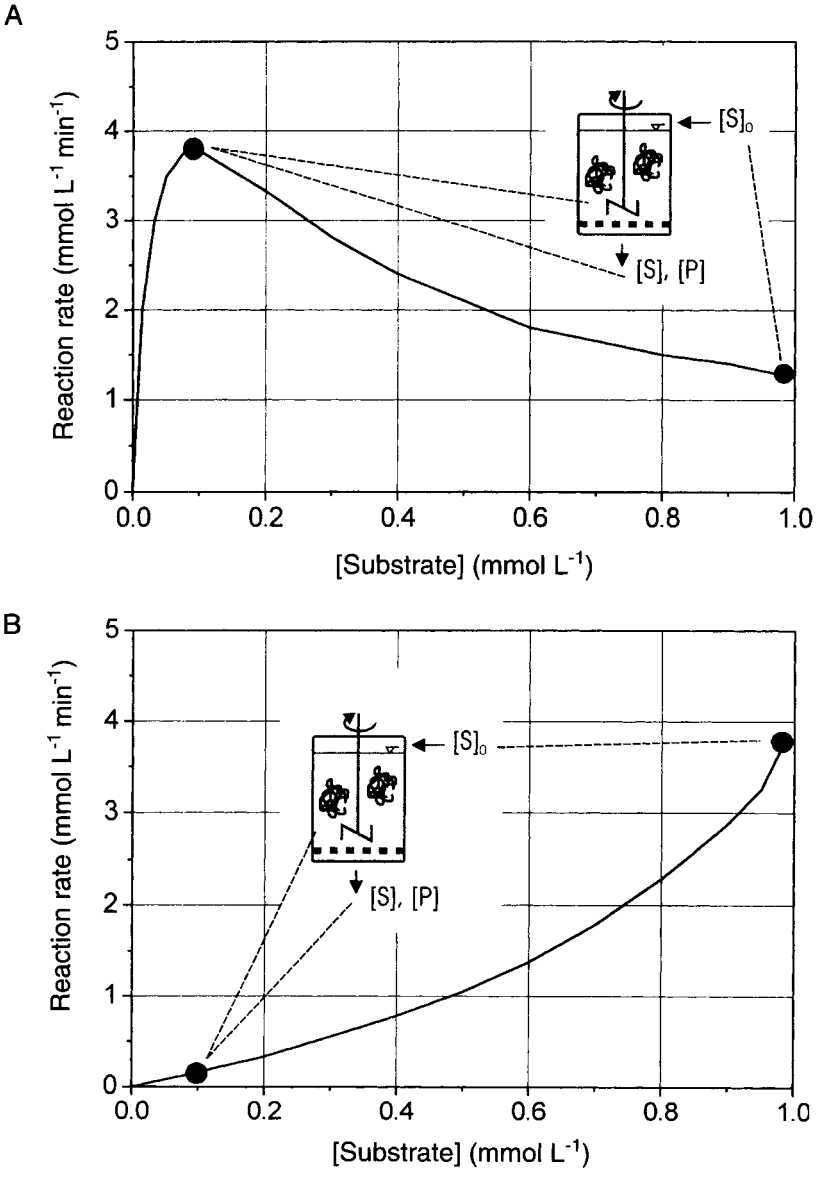


Figure 7-24. Performance of a CSTR depending on enzyme kinetics: **A** substrate inhibition, and **B** product inhibition (bold points indicate the inlet and outlet concentrations of a CSTR).

- The ideal reactor to overcome substrate inhibition (Fig. 7-24 A) is the continuous stirred tank reactor (possible in form of an Enzyme Membrane Reactor, see below). In spite of a high feed concentration of substrate a high reaction rate occurs, as the steady state substrate concentration within the reactor is low.

- In the case of product inhibition (Fig. 7-24 B), the continuous stirred tank reactor is not beneficial, as a high reaction rate would occur at high substrate concentration and low conversion only. With increasing conversion the inhibiting effect of the product becomes dominating, yielding a reduced reaction rate. The CSTR as a whole operates at low steady state substrate concentration and therefore low reaction rate.

In the case of product inhibition, the plug flow reactor is advantageous, as the concentration of the inhibiting product slowly increases along the reactor length, whereas in a CSTR the product concentration is at the maximum value in the whole reactor. Therefore, the average reaction rate is higher in the PFR.

A plug flow reactor may be realized using immobilized enzymes within a column reactor or using soluble enzymes within a cascade of membrane reactors. A batch or a repetitive batch process with soluble enzymes (see below) has the same productivity as the plug flow reactor.

The overall reaction rate not only determines the necessary reaction time to reach the desired conversion (see Fig. 7-23) but also the enzyme and cofactor consumption, both being strongly influenced by the reactor conditions.

Enzyme and cofactor consumption can be defined as follows:

- Enzyme consumption is defined as the number of units of enzyme consumed per unit weight of product ($U\,kg^{-1}$);
- Cofactor consumption is specified with the "total turnover number", defined as mols cofactor consumed per mol of product formed.

As in most cases reaction rate decreases with conversion, enzyme consumption increases to the same extent (an exceptional case is when strong substrate inhibition overcompensates product inhibition). Therefore enzyme consumption is minimal under initial reaction rate conditions (zero conversion) (Fig. 7-25). Approaching total or equilibrium conversion the reaction rate approaches zero and enzyme consumption increases rapidly.

In contrast to enzyme consumption, substrate utilization, defined as kg substrate consumed per kg product produced, increases with increasing steady state conversion.

Besides the classical engineering question of reactor choice, the most important point in enzyme reactor design is the aspect of enzyme reuse, either by immobilization or by separation from the product stream. Batch processes without enzyme reuse are only possible if the costs of the biocatalyst are negligible. Different reactor techniques addressing the aspect of enzyme reuse are discussed in the following sections.

7.5.2
Reactors for Soluble Enzymes

If soluble enzymes exhibit sufficient operational stability, their use is advantageous, as the effort of immobilization and resulting mass transfer limitations can be avoided. Different techniques have been developed to retain soluble enzymes.

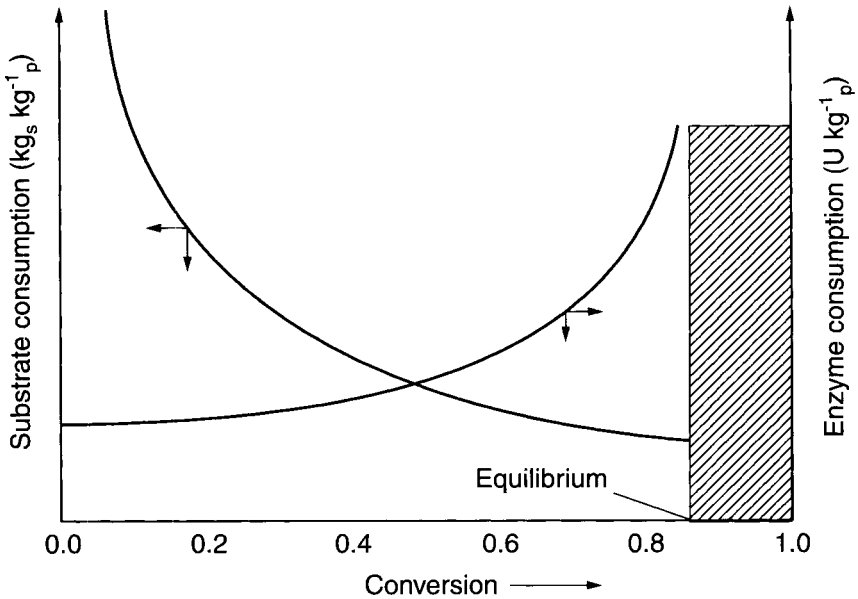


Figure 7-25. Substrate utilization and enzyme consumption as a function of conversion.

For small-scale synthesis enclosure of enzymes in dialysis tubes has been described for several systems (membrane-enclosed enzyme catalysis or the MEEC technique[127]). In this case mass transport of the low-molecular-weight substrates and products across the membrane becomes rate limiting because mass transport only occurs by diffusion and not by convection as described below.

For synthesis on a preparative scale, repetitive batch processing has proved to be an effective and easy-to-handle method[128]. The repeated use of the enzyme is possible after concentration of the solution by means of commercially available ultrafiltration equipment and adding fresh substrate solution. Some of the advantages given for the Enzyme Membrane Reactor (see below) are also valid for the repetitive batch technique.

Compared to batch processes, continuous processes often show a higher space-time yield. Reaction conditions may be kept within certain limits more easily. For easier scale-up of some enzyme-catalyzed reactions, the Enzyme Membrane Reactor (EMR) has been developed. The principle is shown in Fig. 7-26 A. The difference in size between a biocatalyst and the reactants enables continuous homogeneous catalysis to be achieved while retaining the catalyst in the vessel. For this purpose, commercially available ultrafiltration membranes are used. When continuously operated, the EMR behaves as a continuous stirred tank reactor (CSTR) with complete backmixing. For large-scale membrane reactors, hollow-fiber membranes or stacked flat membranes are used[129]. To prevent concentration polarization on the membrane, the reaction mixture is circulated along the membrane surface by a low-shear recirculation pump (Fig. 7-26 B).

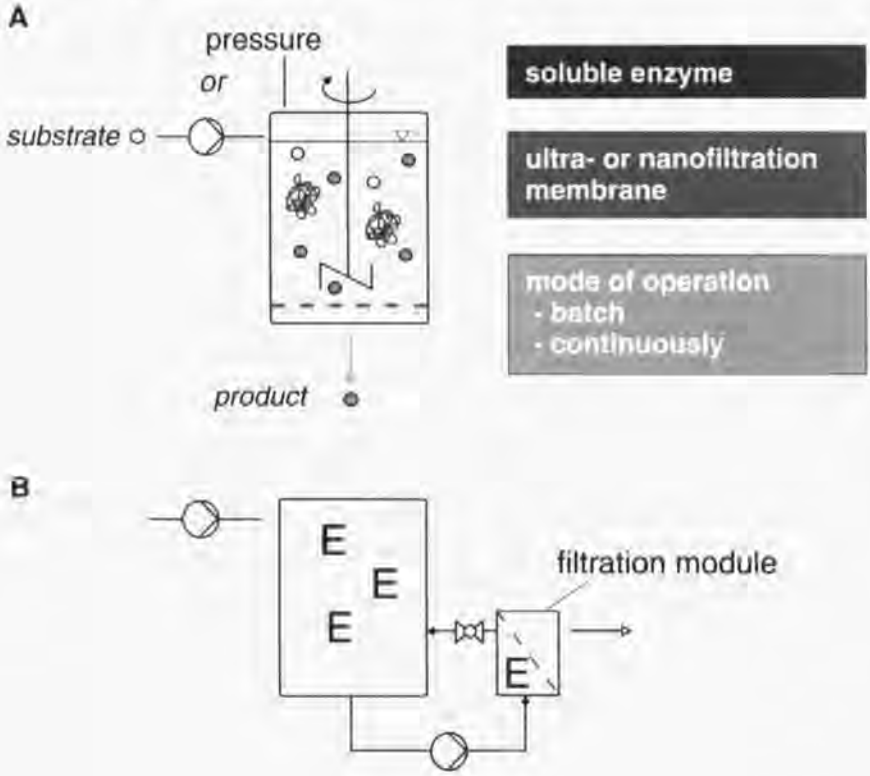


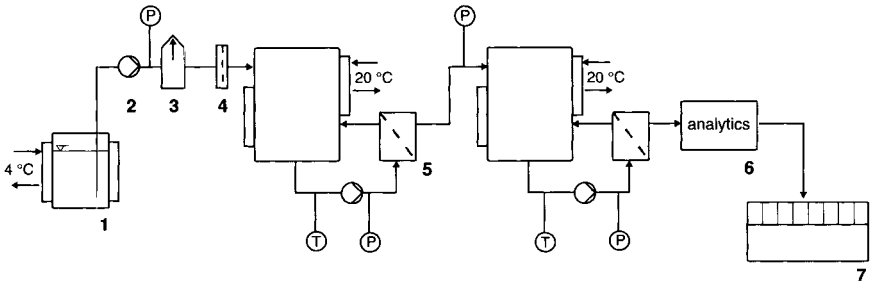
Figure 7-26. **A** Principle of the Enzyme Membrane Reactor. **B** Set-up for larger scale including pump for circulation and filtration module.

Advantages and disadvantages of the EMR are summarized as follows:

Advantages
- Working under sterile conditions is easy to achieve;
- No loss of enzyme activity by immobilization;
- No mass transport limitation;
- High volumetric activity achievable;
- Use of multi-enzyme systems without mass transport limitation;
- Use of coenzymes without mass transport limitation;
- Simple addition of fresh enzyme to compensate for enzyme deactivation;
- Ultrafiltered (pyrogen-free) product solution.

Disadvantages
- Sometimes limited by enzyme stability in solution;
- Back-mixing is not optimal for enzymes with product inhibition.

Figure 7-27 shows a flow chart of the experimental set-up of a two-stage membrane reactor cascade. A 10 mL version of the reactor for process development and small-



Ⓟ pressure gauge
Ⓣ thermometer
1 substrate reservoir
2 pump (dosing, circulation)
3 bubble trap
4 sterile filter
5 ultrafiltration module
6 on-line analytics (pH, UV, polarimeter u. a.)
7 fraction collector

Figure 7-27. Flow chart of the experimental setup of the EMR.

scale production has been commercialized[130]. Depending on the reactor and membrane material, sterilization by means of steam or chemicals (peracetic acid) is possible.

7.5.2.1
Reactor Optimization Exemplified by the Enzyme Membrane Reactor

The performance of the EMR may be calculated by means of the measured kinetics and the simultaneous calculation of mass balances of each reactant. The steady-state parameters of the reactor can be estimated by numerical integration of the differential mass-balance equations by means of the Runge-Kutta method.

Simulation of the reactor performance is a useful tool to find suitable conditions for production. Therefore it is a method resulting in a saving of both time and costs by avoiding large numbers of different experiments. But it has to be proven that the model is able to describe the process within the range of interest.

In the following discussion the method of reactor optimization will be demonstrated using two enzyme systems introduced earlier, namely the enzymatic synthesis of *N*-acetylneuraminic acid and the enzymatic synthesis of cyanohydrins using oxynitrilase.

Example: Enzymatic synthesis of N-acetylneuraminic acid
This example has been used earlier to discuss the influence of thermodynamics on the reaction conditions (Fig. 7-7). It was shown that a great excess of pyruvate is helpful in order to increase equilibrium conversion, but for practical reasons there is a limit, because

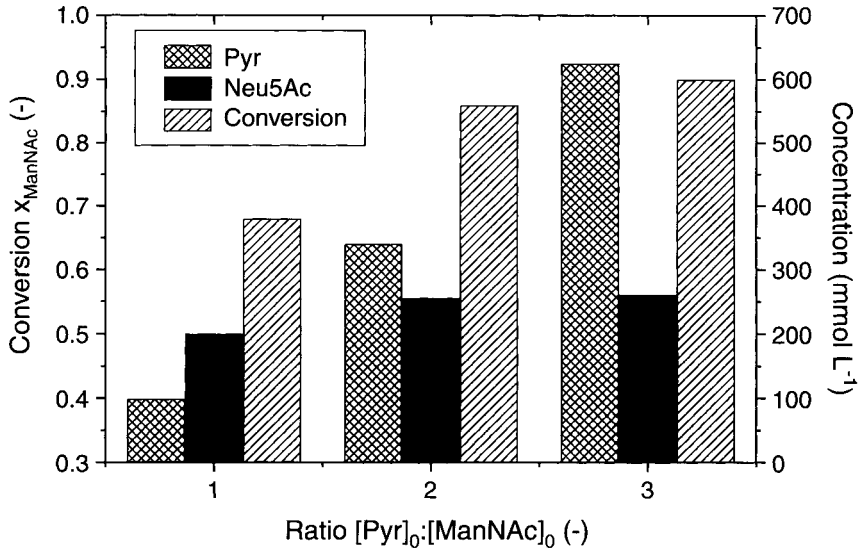


Figure 7-28. Steady-state concentration and conversion as functions of initial substrate ratio in an EMR [47]; conditions: $[E]_0 = 4$ g L^{-1}, τ=3 h, $[ManNAc]_0 = 300$ mmol L^{-1}.

- to reach 91% conversion instead of 86% the amount of pyruvate has to be increased by 50% (conditions specified in Fig. 7-28);
- with increasing concentration of pyruvate, downstream processing is more difficult and time consuming (Neu5Ac and pyruvic acid have similar pK_a values (2.0 and 2.4), complicating separation by anion exchange chromatography).

To enable a quantitative description of the system, kinetic measurements of synthesis and cleavage of Neu5Ac were performed and expressed by a kinetic model [47]. The overall reaction rate is given by Eq. (66).

$$\upsilon = \frac{d[P]}{dt} = \frac{[E]_0 \cdot \left(\frac{A_{S,max} \cdot [A] \cdot [B]}{K_I^A \cdot K_M^P} - \frac{A_{C,max} \cdot [P]}{K_M^P} \right)}{\left(1 + \frac{[A]+[K]+[P]}{K_V}\right) \cdot \left(1 + \frac{[A]}{K_I^A} + \frac{K_M^A \cdot [B]}{K_I^A \cdot K_M^B} + \frac{[A] \cdot [B]}{K_I^A \cdot K_M^B} + \frac{[P]}{K_M^P} + \frac{[P] \cdot [B]}{K_I^B \cdot K_M^P}\right)} \tag{66}$$

Abbreviations: A=Pyr, B=ManNAc, P= Neu5Ac, S=synthesis reaction, C=cleavage reaction.

υ	(kat L^{-1})	Velocity of Neu5Ac synthesis
$A_{S,max}$	(kat g^{-1})	Maximum specific activity of synthesis
$A_{C,max}$	(kat g^{-1})	Maximum specific activity of cleavage
K_M^A	(mol L^{-1})	Michaelis-Menten constant of Pyr
K_M^B	(mol L^{-1})	Michaelis-Menten constant of ManNAc
K_M^P	(mol L^{-1})	Michaelis-Menten constant of Neu5Ac
K_I^A	(mol L^{-1})	Inhibition constant of Pyr

Table 7-4. Kinetic parameters of Neu5Ac-aldolase.

Synthesis of Neu5Ac			Cleavage of Neu5Ac		
$A_{S,max}$	230.5	μkat g^{-1}	$A_{C,max}$	141.8	μkat g^{-1}
	13.8	U mg^{-1}		8.51	U mg^{-1}
K_M^A	0.136	mmol L^{-1}	K_M^P	9.44	mmol L^{-1}
K_M^B	402.2	mmol L^{-1}	K_I^A	1.30	mmol L^{-1}
			K_I^B	23.8	mmol L^{-1}
K_V	1556	mmol L^{-1}			

K_I^B	(mol L^{-1})	Inhibition constant of ManNAc
$[E]_0$	(g L^{-1})	Enzyme concentration (aldolase)
K_V	(L mol^{-1})	Concentration-dependent inhibition

The first term in the denominator of Eq. (66) represents a non-competitive inhibition (compare to Eq. (31)) of the enzyme by the sum of concentrations of A, B and P. This non-specific inhibition could be correlated with an increased viscosity of the reaction medium in the presence of A, B and P[47, 131]. As the mutarotation of the carbohydrates is fast compared to the enzymatic reaction there was no need for a discrimination between the α,β-anomers or the open-chain form of the monosaccharides. A complete set of kinetic parameters was determined, summarized in Table 7-4.

The mass balances for the substrates and products are given by Eqs. (67–69).

$$\frac{d[\mathrm{Pyr}]}{dt} = \frac{[\mathrm{Pyr}]_0 - [\mathrm{Pyr}]}{\tau} - \upsilon \tag{67}$$

$$\frac{d[\mathrm{ManNAc}]}{dt} = \frac{[\mathrm{ManNac}]_0 - [\mathrm{ManNAc}]}{\tau} - \upsilon \tag{68}$$

$$\frac{d[\mathrm{Neu5Ac}]}{dt} = \frac{[\mathrm{Neu5Ac}]_0 - [\mathrm{Neu5Ac}]}{\tau} + \upsilon \tag{69}$$

At steady state the mass balance of each component N may be rewritten as shown in Eq. (70).

$$\frac{d[\mathrm{N}]}{dt} = 0 \Rightarrow \upsilon = \frac{[\mathrm{N}]_0}{\tau} \cdot x \tag{70}$$

meaning that the reaction term is equal to the convection term (compare to Eq. (59)). This identity can be used to determine the conversion of the substrate at a given residence time from a plot $\upsilon = f(x)$ (Fig. 7-29).

The point of intersection of the function $\upsilon = f(x)$ and the straight line $\upsilon = [N]_0/\tau \cdot x$ (the "convection term") determines the operating point of the reactor at given concentration $[N]_0$ and residence time τ. The operating point is characterized by substrate conversion and reaction rate under steady-state conditions. By changing the slope of the convection line, e.g. by change of the residence time, other operation

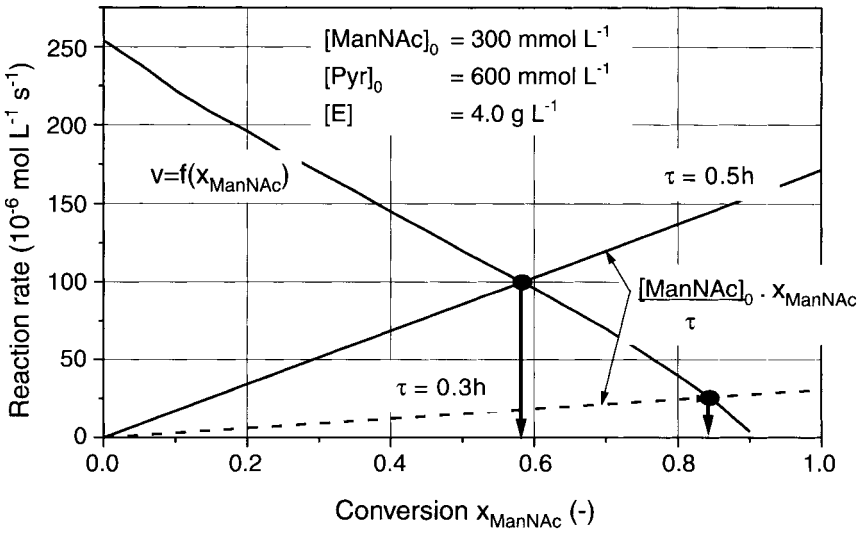


Figure 7-29. Graphical method of determination of steady-state operation conditions in an EMR[47] (bold points indicate steady-state reactor conditions; asterisks indicate steady-state conversion).

points may be determined. Instead of increasing the residence time the enzyme concentration may be increased to the same extent, yielding the same result (compare to the [E] · τ concept).

This method enables determination of operation conditions of an EMR from batch reactor data. For that purpose a differentiation of the function $x(t)$ has to be performed and converted to the above curve $v = f(x)$.

Using the mass balances, optimum operating conditions for continuous production in the EMR were calculated. Concentrations of 300 mmol L^{-1} ManNAc and 600 mmol L^{-1} pyruvate were found to be the most suitable to allow high conversion of ManNAc, high space-time yield, and easy product isolation. Figure 7-28 shows steady-state concentration and conversion as a function of substrate ratio in an EMR.

With a ManNAc concentration of 300 mmol L^{-1} and an equimolar amount of pyruvate in the feed, 68% conversion is reached and the product solution contains 200 mmol L^{-1} Neu5Ac, 100 mmol L^{-1} pyruvate and 100 mmol L^{-1} ManNAc. At this low conversion, unreacted ManNAc should be recovered, requiring an additional purification step. Conversion increases with excess of pyruvate to almost 90% at only twofold excess of pyruvate, but there is a larger amount of pyruvate remaining in the product solution which has to be separated.

In Fig. 7-30, conversion and space-time yield for the continuous process in an EMR are depicted. Enzyme concentration and residence time were chosen to be within practical limits to ensure a reasonably high conversion, suitable for production.

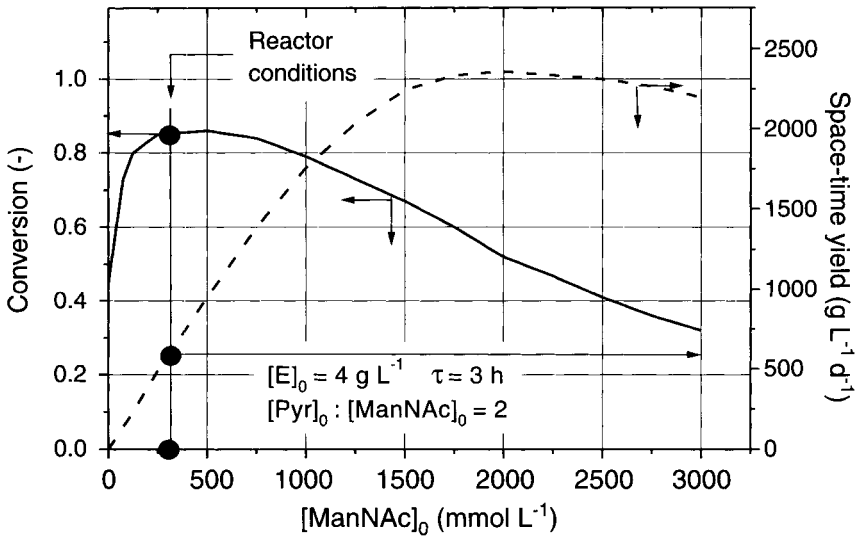


Figure 7-30. Conversion and space-time yield as function of substrate concentration[47].

The increase of conversion with rising concentration results from an increase of equilibrium conversion as depicted in Fig. 7-7. The decrease of conversion at ManNAc concentrations >500 mmol L^{-1} is due to the increasing effect of non-specific inhibition (compare to Eq. (66)). The space-time yield decreases when the decrease of conversion exceeds the increase of ManNAc-concentration.

According to these results a feed concentration of 300 mmol L^{-1} ManNAc was chosen for Neu5Ac production, allowing a high conversion (>85%) and a good space-time yield (650 g $L^{-1}d^{-1}$). The latter may be raised by increasing enzyme concentration and decreasing residence time ([E] · τ=constant), resulting in the same conversion within a shorter time.

The model proved to be correct by a comparison of predicted and experimental conversions for several enzyme concentrations and residence times (Fig. 7-31).

Biocatalyst consumption per unit weight of product was found to be about 6000 U/kg at a conversion of 78%. For production purposes, enzyme membrane reactors with a working volume up to 500 mL were employed for the synthesis of approximately 2 kg of *N*-acetylneuraminic acid and other derivatives such as keto-desoxynonulosonic acid (KDN)[129, 132]. Downstream processing was achieved mainly by anion exchange chromatography on a 30 L column followed by reverse osmosis to concentrate solutions before lyophilization.

Example: Enzymatic synthesis of benzaldehyde cyanohydrin

In the case of the enzymatic synthesis of cyanohydrins, enantioselectivity is the most important criterion. Investigation of the kinetics of the whole system (enzymatic and non-enzymatic reaction) offers the possibility to optimize reaction conditions to

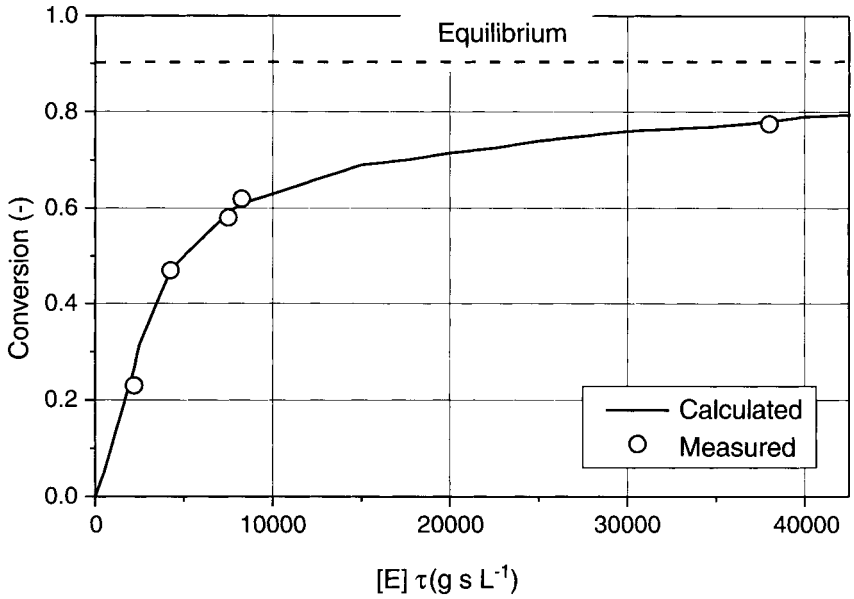


Figure 7-31. Comparison of calculated and experimental conversion [47].

obtain products with high optical purity. The discussion within Sect. 7.3.2.2 is extended to a consideration of reactor design.

From the kinetics of the enzymatic and the non-enzymatic reactions (Fig. 7-13) it is concluded that the side-reaction is suppressed very effectively by working with high enzyme concentrations and at a low benzaldehyde concentration. Benzaldehyde may react with amino functions of the enzyme to form Schiff bases resulting in deactivation of oxynitrilase, so low stationary benzaldehyde concentrations are also necessary with respect to enzyme stability.

As the EMR behaves as a CSTR, conversion in the whole reactor becomes high and the stationary benzaldehyde concentration becomes low if the product $[E] \cdot \tau$ is chosen to be sufficiently high.

Enzyme concentration as well as residence time τ have been varied and the resulting conversion and enantioselectivity calculated. Fig. 7-32 shows enantioselectivity as a function of conversion in continuous experiments.

Different operating conditions were obtained by changing the enzyme concentration. The benzaldehyde concentration is determined by its maximum solubility. A fourfold excess of hydrogen cyanide is necessary to reach conversions greater than 90%. A larger excess will favor the non-enzymatic reaction yielding lower enantioselectivities. As already postulated from the initial rate measurements, enantioselectivity increases with increasing conversion. There is a good correlation between calculated and measured values, as well as for other substrate ratios not shown here. The reactor conditions and results are presented for one experimental run.

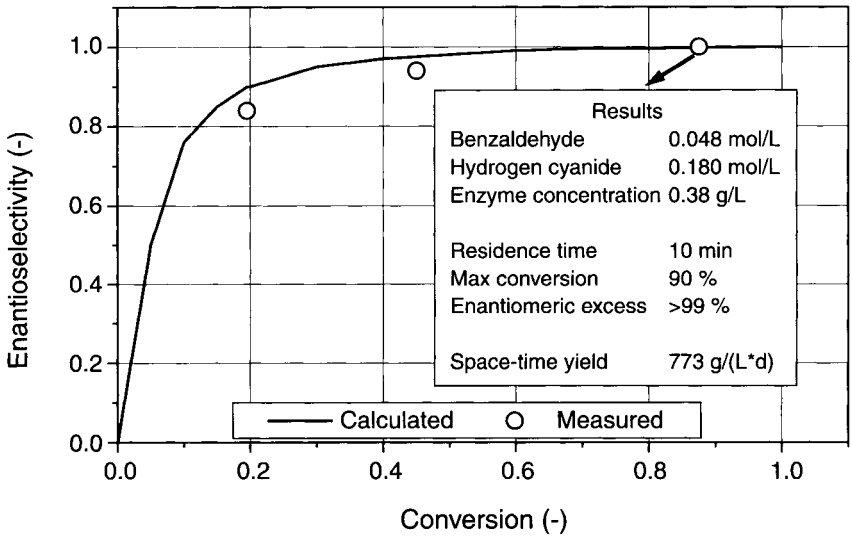


Figure 7-32. Enzymatic synthesis of benzaldehyde cyanohydrin in an EMR: calculated and experimental enantioselectivity as a function of conversion[55, 60].

In a production run, the space-time yield was enhanced to 2400 g $L^{-1}d^{-1}$ *(R)*-mandelonitrile (*ee*>99 %) at a residence time of just 3.8 min using an enzyme concentration of 0.95 g L^{-1}.

A similar investigation for batch reactor systems has been published recently[133]. DSM Linz in Austria established a process for the synthesis of (S)-phenoxybenzaldehyde cyanohydrin using a cloned (S)-oxynitrilase[59, 134]. The reaction is performed in a two-phase system with the substrate dissolved in an organic solvent.

Example: Coupled enzyme systems

In Sect. 7.4.3, the reduction of dihydroxyphenylpyruvic acid (DHPP) to dihydroxyphenyllactic acid (DHPL) was used as an example for discussion of the kinetics of multiple enzyme systems (Eq. (49)). The rate equations for the reduction reaction of DHPP to DHPL (ν_1) and the regeneration of PEG-NAD^+ to PEG-NADH (ν_2) have been introduced (Eqs. (50) and (51)).

The mass balances of all components in the case of performing the reaction in a CSTR are formulated as follows (Eqs. (71)-(75)):

$$\frac{d[DHPP]}{dt} = \frac{[DHPP]_0 - [DHPP]}{\tau} - \upsilon_1 \tag{71}$$

$$\frac{d[DHPL]}{dt} = \frac{[DHPL]_0 - [DHPL]}{\tau} + \upsilon_1 \tag{72}$$

$$\frac{d[For]}{dt} = \frac{[For]_0 - [For]}{\tau} - \upsilon_2 \tag{73}$$

$$\frac{d[\text{PEG-NADH}+\text{H}^+]}{dt}=\upsilon_2-\upsilon_1 \tag{74}$$

$$\frac{d[\text{PEG-NAD}^+]}{dt}=\upsilon_1-\upsilon_2 \tag{75}$$

In the case of PEG-NAD$^+$ and PEG-NADH, the convection term equals zero as both are completely retained within the reactor by the ultrafiltration membrane. The enzymatic reactions are coupled by PEG-NADH and PEG-NAD$^+$. As stated previously, this stoichiometric coupling does not affect the enzyme kinetics, but has to be considered when writing the mass balances.

Considerations about process optimization of coupled systems with coenzyme regeneration are discussed in the literature[29, 42,43, 135]. One aspect may be illustrated here – the question of enzyme ratio within the coupled enzyme system.

Figure 7-33 represents the influence of the ratio of the two enzymes D-HicDH for the synthesis reaction and FDH for the cofactor regeneration.

The enzyme ratio represents the ratio of the initial volumetric activities of the enzymes (dimensions U mL^{-1}). For process conditions, enzyme activity under the actual steady-state reactor conditions is significant, and differs from initial rate conditions as determined by enzyme kinetics. Therefore, the optimum enzyme ratio, implying maximum conversion within minimum residence time, is not 0.5.

As evident from Fig. 7-33, it does not make sense to add one enzyme, e.g. the cheaper one, in excess to the reaction mixture, as, a long way from the optimum of

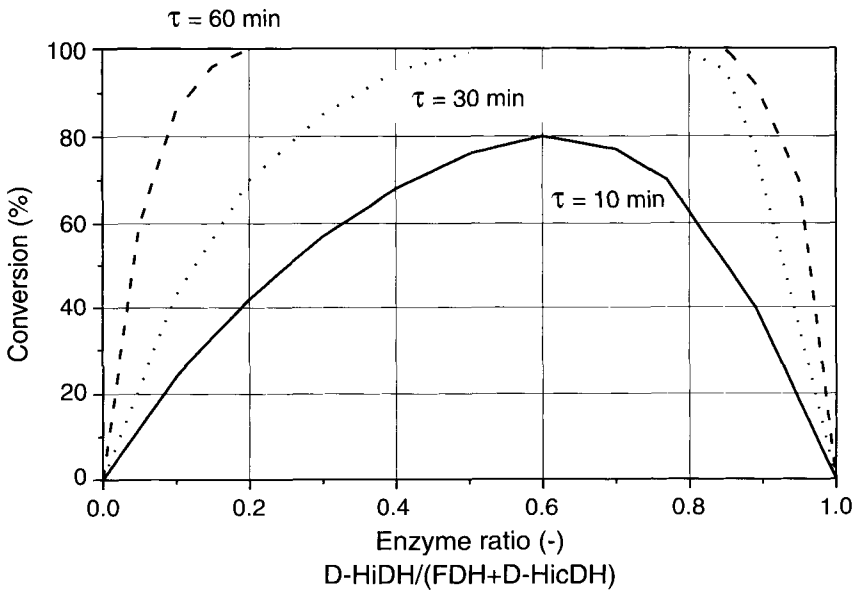


Figure 7-33. Dependence of conversion on enzyme ratio at different residence times[119].

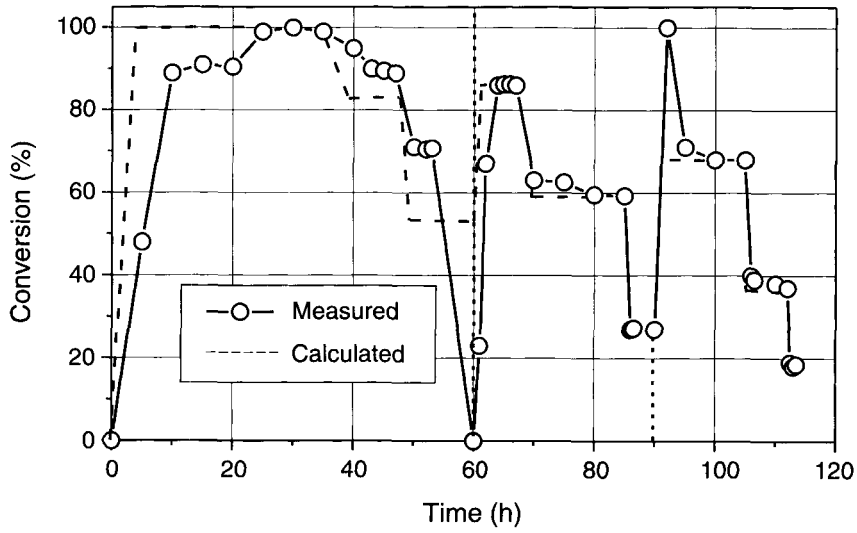


Figure 7-34. Comparison of calculated and measured data for the enzymatic synthesis of dihydroxyphenyllactic acid in an EMR[119].

enzyme ratio, only one enzyme determines the overall reaction rate. This situation might appear in coupled enzyme assays where care has to be taken that the enzyme of interest is rate determining.

As proof of the kinetic model, fitting of initial rate data or time-course data of batch reactions have been introduced in Sect. 7.4. Additionally, a proper fit of continuous reactions in an enzyme membrane reactor (EMR) may serve as confirmation of the kinetic model. For this coupled enzyme system, calculated and measured conversions at different operating conditions (varying [E] and τ values, not further specified) are presented in Fig. 7-34.

7.5.2.2
Control of Conversion in a Continuously Operated EMR

As a consequence of enzyme deactivation, conversion may drop during the continuous operation of enzyme reactors. To maintain a constant degree of conversion, two methods can be employed according to the [E] · τ-concept (see above):

- addition of fresh enzyme,
- reduction of flow rate.

Both can be done very effectively by using methods of online analytics combined with an appropriate automatic controller. Useful methods for online analysis of enzymatic processes are

- polarimetry (useful for reactions where chiral reactants are involved)[136],
- UV spectrometry,

- online-HPLC (may be used effectively for controlling complex reactions (e.g in peptide or carbohydrate synthesis)) [137].

7.5.3
Reactor Systems for Immobilized Enzymes

The choice of an appropriate reactor for applications of immobilized enzymes as well as for soluble enzymes depends on the kinetics of the reaction. Kinetics of immobilized enzymes are not only a function of enzyme activity but also of substrate transport to the enzyme, which is affected by the matrix used for immobilization. For a description of immobilized enzyme kinetics the reader is referred to the comprehensive literature in this field [35–40, 138–140]. Additionally, the use of immobilized enzymes is treated in Chap. 6 of this book.

A brief overview of enzyme reactors used for application of immobilized biocatalysts in the laboratory and on the industrial scale is given in Fig. 7-35. Examples of industrial processes are given in [2] and [20].

To study the kinetics of immobilized enzymes a *recirculation reactor* may be used. This reactor allows one to perform kinetic measurements with defined external mass transfer effects, reached by establishing a high flow rate near the catalyst, minimizing mass transfer resistance. The reactor behaves as a differential "gradientless" reactor allowing initial-rate kinetic measurements to be made.

The *fixed bed reactor,* behaving as a plug flow reactor, is most often used for immobilized enzyme reactions. Typically, the reactor is used with an upward direction of the flow to avoid compression of the bed and to release gas bubbles generated during the reaction. Reactor design may be done readily without knowing the detailed enzyme kinetics. Kinetic measurements are performed with a recirculation reactor and the data are plotted in the form $1/v = f(x)$ (see above). From this plot, the residence time necessary to reach a desired conversion x can be calculated as described. The different enzyme concentrations in the recirculation reactor and in the plug flow reactor have to be considered.

Fluidized bed reactors are advantageous if small particles which would give high flow resistance in a fixed bed reactor are used to minimize external mass transfer resistances. Often it is useful to install nets at different heights of the reactor to approach plug flow characteristics.

If the immobilized enzyme particle size is so small that an effective retainment is not possible in fluidized bed reactors, a *slurry reactor* may be used. This reactor guarantees catalyst retainment using a filter or a microfiltration membrane. For a larger particle size, the use of a stirred tank reactor is not advantageous because the energy input necessary to give an optimal fluidization of the particles is much higher than in a fluidized-bed type reactor.

Besides the immobilization of enzymes on solid particles, enzymes may also be immobilized on the inner or outer surface of tubular supports such as on hollow fibers or flat membranes. Enclosure of enzymes by the use of an ultrafiltration or dialysis membrane is regarded as a form of immobilization.

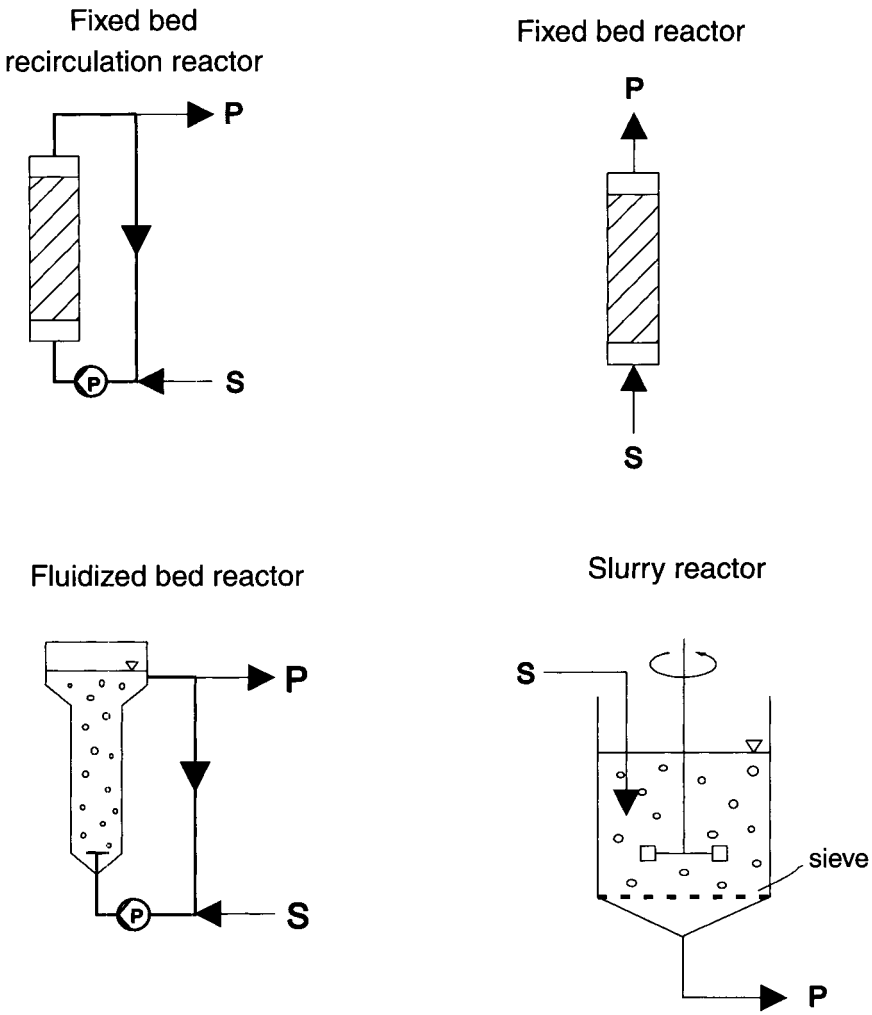


Figure 7-35. Reactors for use of immobilized enzymes.

7.5.4
Reaction Techniques for Enzymes in Organic Solvents

The same reactors can be used for dealing with immobilized enzymes in organic solvents or with one-phase organic systems as for dealing with enzymes in aqueous solutions. For one-phase systems, the enzyme may be recovered from the solution by means of membrane filtration. Suspended enzyme particles may be retained in a slurry reactor (compare to Fig. 7-35) by microfiltration membranes or stainless steel sieves, whereas in other cases such as reverse micelles, ultrafiltration membranes have to be used[89]. For some years ultrafiltration membranes have been available

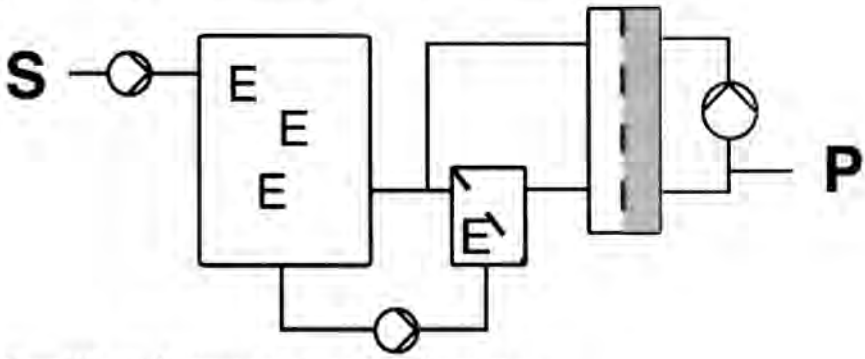


Figure 7-36. Multi-compartment enzyme membrane reactor.

which are stable toward organic solvents (e.g. from polyaramide or cellulose). In these cases the enzyme membrane reactor, as described earlier for the pure aqueous system, may be used without modifications, if all materials (sealing rings, tubes) are stable toward the solvent used.

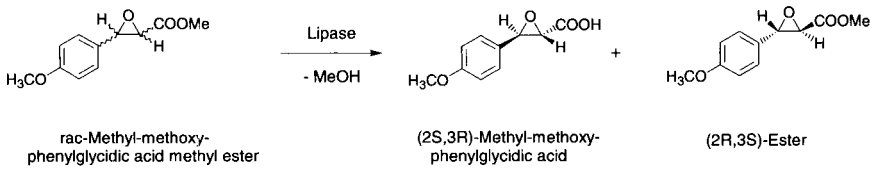
For two-phase systems, special reactors may be used such as multi-compartment reactors as shown in Fig. 7-36[43, 141, 142].

In the latter case, the two phases are separated by a hydrophobic or hydrophilic membrane (solid supported interface). The enzyme is soluble in the aqueous phase and substrate is added up to its maximum solubility in the aqueous phase. Substrates and products are distributed according to their hydrophilic or hydrophobic properties. The membrane area has to be large enough to avoid mass transfer limitations. High membrane areas may be achieved by flat membrane stacks or by hollow fiber modules.

If lipases are used they may be adsorbed at the interface on a hydrophobic membrane. Such a system has been developed by Sepracor, USA, for the enantioselective hydrolysis of racemic esters (Fig. 7-37) and is used by Tanabe Seiyaku Co. for the kinetic resolution of 3-(4-methoxyphenyl)glycidic acid methyl ester, which is an intermediate in the synthesis of diltiazem[20,77,143].

Here the racemate is circulated on one side of the membrane while the water necessary for hydrolysis is picked up from the other side. The resulting acid is extracted into the aqueous phase, where the pH is kept constant with NaOH. The enzyme is adsorbed in the pores of the membrane. For the pilot scale production the following data were obtained: reactor productivity 125 g $d^{-1}m^{-2}$, enzyme consumption 17 g per kg product, enantiomeric excess of product 99 %, product yield 43 %.

Additional reactor systems, especially reactors for synthesis of oleochemicals are summarized in [99].



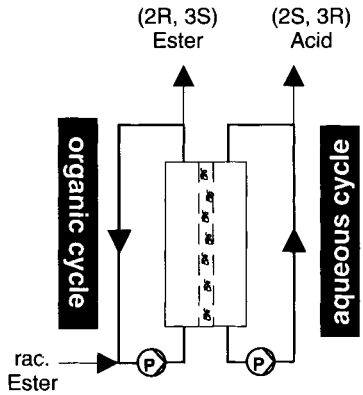


Figure 7-37. Membrane reactor system for resolution of a diltiazem intermediate.

7.6 Conclusions and Outlook

Some basic aspects of reaction engineering for enzyme-catalyzed biotransformations have been presented within this chapter, using examples of processes investigated by the authors. A strategy was shown starting with considerations focusing on the appropriate enzymes, continuing with an investigation of enzyme kinetics, and developing a reactor model. Finally, with the aid of process optimization, the most favorable reactor conditions can be found in order to achieve a high space-time yield with high conversion, high selectivity and low enzyme consumption.

Enzyme engineering has reached a status where many limitations have been overcome by interdisciplinary efforts. New enzymes are being sought and, indeed, found in natural environments which will perform biotransformations on unnatural substrates. Huge quantities of enzymes can be produced by genetic engineering methods, making availability and costs of enzymes no longer a barrier for commercial processes. Much progress has been made in the evolution of biocatalysts to improve their properties. But still the choice of solvent to perform the reaction and the configuration of the surrounding reactor are important issues to be considered as well, to obtain optimum results.

Based on enzyme kinetics well established by biochemists, complex reactions can be modeled. By utilizing classical reaction engineering methods, a quantitative

description of process performance can be achieved and an operational status of a reactor can be calculated, saving experimental effort. Of particular note is the development of continuous processes, which have the advantage of better controllable reaction conditions, allowing suppression of undesired side-reactions and yielding better selectivity. Space-time yields of several kilograms of product per liter of reactor volume per day have been reached.

The methods of enzyme reaction engineering have already shown their benefits in numerous industrial processes which are being established successfully. Hopefully it can be concluded from this article that process development for the application of enzymes in organic synthesis can be performed on a rational basis.

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8
Enzymic Conversions in Organic and Other Low-Water Media

Peter Halling

8.1
Introduction

It is now often preferred to carry out enzyme-catalyzed biotransformations in non-aqueous media. These are commonly based on organic solvents, but a variety of other low-water media can be useful. Popular reasons for using such systems are listed in Table 8-1.

When a low-water biotransformation is planned, a variety of choices must be made about the precise reaction conditions to be used. As in all biocatalytic systems, the rate and yield obtained can be greatly affected by the choices made. Some of the factors that must be considered are the same as in conventional aqueous media: temperature, reactant concentrations, the form in which the enzyme is added. New factors must be taken into account such as solvent selection and the level of residual water in the system. Other factors become somewhat modified: acid-base conditions remain important, but, usually, pH is no longer a useful parameter to characterize them.

This chapter is written primarily for a reader who wishes to carry out a low-water biotransformation, and requires some general advice on the selection of reaction conditions. It will be a long time before our understanding of these systems is sufficient to predict confidently the optimal conditions for a novel reaction. Of course, we cannot do this for an aqueous biotransformation, or, for that matter, most non-enzymic chemical transformations. However, it is possible to give recommenda-

Table 8-1. Common reasons for choosing low-water media for biotransformations.

Reactants don't have to be (very) water soluble – use different solvent, or just undissolved solids
Changes in solvation alter equilibria and kinetics – e.g. readily available hydrolytic enzymes catalyse synthetic reactions (including direct reversal of hydrolysis)
Can tune specificity by changing medium etc.
Enzymes can be *more* stable
Suppression of unwanted processes in aqueous solution, e.g. microbial growth, side-reactions
Better integration of biocatalytic with chemical steps in non-aqueous media

tions that will often lead to a reasonable performance at first attempt, and will guide the design of experiments to approach closer to the optimum.

In general I will not discuss in detail the evidence that lies behind the recommendations presented here. The reader who wishes to explore this further should consult the following list of recent reviews of the field [1–17]. These form a basis for an individual assessment of the most useful choices. This chapter is more aimed at those who wish to rely on my judgments and is written in a rather brief and prescriptive style in many places, in order to minimize the length.

Undoubtedly some of these recommendations will be found less than ideal in the light of further investigation. However, I am fairly confident that most of them will remain good choices.

8.2 Enzyme Form

In aqueous media, the most usual state of the enzyme molecules is dissolved in the reaction medium. In this case, the previous treatment of the enzyme has little effect on catalytic activity, provided irreversible inactivation is avoided. In contrast, in low-water media, the enzyme molecules are usually present in solid particles. The way the solid biocatalyst is prepared will clearly affect the state of the enzyme molecules, and hence their catalytic properties. Furthermore, as hydration of the enzyme molecules is reduced, it is clear that conformational changes can become much slower. As a result the previous history of the enzyme has important effects, not just the final conditions. In other words, there may be pronounced hysteresis effects.

Enzymes are normally isolated from their biological source as an aqueous solution. Hence preparation for transfer to a low-water medium requires removal of much of the water from the environment of the protein molecules. A variety of different drying methods can be considered. Although presented below are certain statements about the relative activity of these different forms, it must be acknowledged that there have been few direct comparisons of the same enzyme prepared in different ways but then used in the same medium.

8.2.1 Lyophilized Powders

Lyophilization (freeze-drying) is one of the most obvious ways of producing solid particles from an enzyme solution, and is probably the most used in preparing low-water biocatalysts. However, I would generally *not* recommend it. It is of course a standard and usually reliable method of drying enzymes for storage. However, lyophilized enzyme powders used directly in low-water media typically show much lower specific activity (per enzyme molecule) than other preparations. At least two factors seem to contribute. Many of the enzyme molecules in lyophilized powders have changed conformations, probably due to the conditions in the concentrated unfrozen regions during freezing. This (partial) denaturation is reversed on return

to aqueous solution, but not in low-water media. Considerable attention has been given to methods that can increase the specific activity of lyophilized powders by orders of magnitude, such as drying in the presence of salts or lyoprotectants. But these large increases reflect primarily the very poor activity of the control lyophilized powders.

8.2.2
Immobilized Enzymes

Immobilization is an alternative route to solid particles containing the enzyme, typically by attachment to a pre-existing support. Immobilized forms of many enzymes are now commercially available. For use in low-water media, the attachment is usually done very simply by adsorption – the support particles are simply stirred for a time in the aqueous enzyme solution. The strength of the linkage to the surface is not really an issue, because enzyme desorption will not normally be possible in low-water media. Much of the water is removed by decanting the supernatant. The wet solid particles may then be dried further by evaporation in air. However, a better method seems to involve rinsing with a suitable solvent that is able to dissolve plenty of water (i.e. usually a water-miscible solvent). This exploits the hysteresis in enzyme behavior to give a higher specific activity catalyst, at least for initial rates and with some enzymes. Drying procedures are discussed further below in the context of water effects.

More complicated immobilization methods have been described, including covalent attachment to a support, entrapment inside particles (such as silica made by a sol-gel process), and covalent incorporation into polymer particles ("biocatalytic plastics"). However, it is not clear that any of these methods is superior to simple adsorption, particularly for use in low-water media, where enzyme desorption is not an issue.

A method first described around 1980 has remained popular – drying a slurry of support material (commonly celite) in an enzyme solution. The resulting powder necessarily contains both solid support and enzyme. However, the link between them is usually very weak, and the method is now usually described as co-drying or deposition rather than immobilization. At least some of the enzyme is probably present as large aggregates, not close to the support. My impression is that catalytic activity is usually poorer than with enzymes immobilized by methods more likely to produce an even protein layer.

8.2.3
Cross-Linked Crystals

One enzyme form that has received considerable attention is based on enzyme crystals. Production of protein microcrystals from aqueous solution is often quite easy, and is increasingly used as a step in the manufacture of enzymes on an industrial scale. (Many people have the impression that protein crystallization is very difficult, but this stems from the problems in growing large near-perfect crystals for

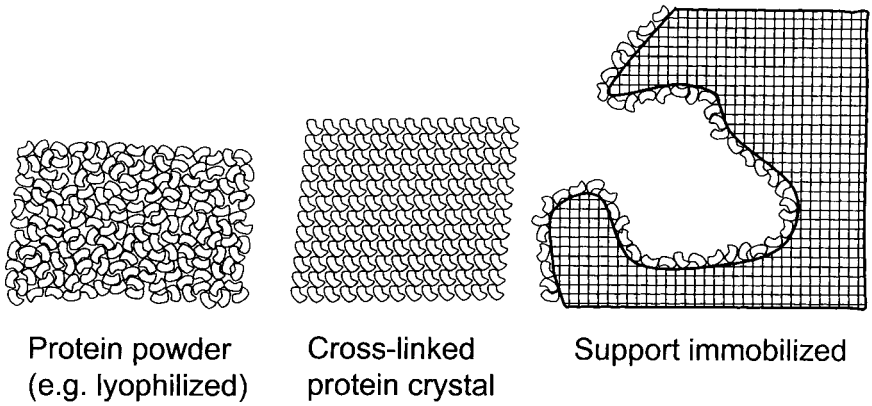

Figure 8-1. Schematic illustration of how enzyme molecules are organized in different biocatalyst forms.

X-ray diffraction.) The protein molecules in the microcrystals are then covalently cross-linked by treatment with an appropriate multi-functional reagent, usually glutaraldehyde. This renders the crystals insoluble on transfer to different aqueous media. The cross-linked crystals are effectively another form of immobilized enzyme, and can be dried for transfer to low-water media by the same methods (again see further details in the discussion of water effects below). Cross-linked crystals are available commercially for a number of enzymes. Figure 8-1 shows a diagrammatic representation of the organization of the protein molecules in lyophilized powders, immobilized enzymes and cross-linked crystals.

8.2.4
Direct Precipitation in Organic Solvents

A good and even simpler method of preparation is available where reaction media are based on an organic solvent that has the ability to dissolve considerable amounts of water (or is water-miscible). An aqueous enzyme solution may be mixed directly with the organic liquid (dry or nearly so). Most of the water in the enzyme solution forms a molecular solution in the organic liquid. The enzyme and other solutes are precipitated, usually as fine particles. The catalytic activity obtained is usually quite good, certainly compared with lyophilized powders. In principle particles containing active enzyme prepared this way might be transferred to other solvents by centrifugation and re-suspension.

8.2.5
Additives in Catalyst Powders

A great deal of attention has been given to co-drying additives with enzymes for low-water biocatalysis. Large improvements in rate as well as alterations in selectivity

have been demonstrated. A variety of mechanisms have been suggested for these effects, including the following.

- Denaturation during the drying process may be prevented ("lyoprotection");
- The additive may select particular conformational microstates that become fixed on drying ("imprinting");
- The environment of the enzyme molecules may be favorably altered, for example by being made highly polar (salt effects).

There are undoubtedly some interesting effects here. However, in general I cannot recommend such approaches at present to those persons looking for practical options for biocatalysis. All the main studies of these effects have been made with lyophilized powders as the final catalyst. As noted above, these usually give a very poor specific activity compared with other forms of the same enzyme. This is probably why it is possible to demonstrate very large enhancements in rate – these are relative to a very low base for the control lyophilized powders. It would be very interesting to see whether these additive effects are found with other low-water catalyst forms. It may be that they have been looked for, not found, and hence the results are not reported. Because of the low base activity of lyophilized powders, it is not clear whether the "enhanced rates" resulting from these various additives are better than (or even as good as) those obtained with other catalyst forms (e.g. immobilized). It would be useful for some studies to make this comparison systematically, with the same enzyme and reaction. Until such a comparison has demonstrated superior performance for the lyophilized powder, I would recommend the use of other catalyst forms as a simpler and more certain route to good catalytic activity. One exception would be where it is wished to exploit the alteration of specificity brought about by additives.

8.2.6
Solubilized Enzymes

All the enzyme forms discussed so far remain as visible suspended solids in organic reaction mixtures. However, it is possible to treat enzymes so that they become solubilized in organic media (or at least no longer form an obvious suspension). The known methods can be classified into three categories, although these are not clearly distinct at the boundaries:

- The enzyme molecule can be covalently modified with organic soluble groups;
- The enzyme can form a non-covalent complex with appropriate agents, usually either surfactants or organic-soluble polymers;
- The enzyme molecules can be contained in the water cores of hydrated reverse micelles or microemulsion droplets.

These systems have been extensively studied, particularly the last. They have considerable attractions for fundamental studies, because the more or less transparent and mobile systems permit spectroscopic studies. Thus catalytic activity may be easily followed by direct spectrophotometric measurement of substrate or product

concentrations. Fluorimetry or spectrophotometry can be use to probe the protein molecules directly. In microemulsions and some of the other systems, it has been clearly shown that the enzyme is molecularly dispersed. In other cases, however, the individual units remain aggregates, often quite large, but dispersed in such a way that the suspension is more or less transparent.

The specific activity of the enzymes is often good, comparable with the best alternative enzyme forms. The enzyme molecules should all be well accessible to the medium, and mass transfer limitations avoided. However, solubilized enzymes have not achieved widespread use by those mainly concerned with applications in synthesis. An extra step is required to separate the enzyme from the final reaction mixture containing the products. It may be even harder to separate solubilising additives, notably surfactants, from the products. Thus I in general would not recommend solubilized enzymes for synthetic applications. One exception is where it is wished to attack polymeric or solid-state substrates, where the enzyme molecules may need to be able to move to contact the substrate, rather than vice versa.

8.3
Residual Water Level

The level of water remaining in these systems usually has major effects on behavior. Some of these phenomena are due to mass action effects of water as a reactant. The equilibria of hydrolysis reactions become more favorable as water levels rise, and normally the rates increase as well. Low-water media are commonly selected in order to use hydrolytic enzymes to catalyze synthetic reactions. Hydrolysis will thus be an undesirable side reaction, or in many cases the direct reverse of what is wanted. There are also cases where hydrolysis reactions are wanted, and hence water mass action should be maximized (while keeping non-aqueous media for reasons of substrate solubility, for example).

Water levels also have important general effects on enzyme behavior. If too little water is present, the catalytic activity of most enzymes falls dramatically. On the other hand, reduction in water levels often leads to an increase in enzyme stability. A decline in catalytic activity at high water levels is also commonly observed, with several possible explanations:

- The rate of the monitored reaction may fall as a result of competition from hydrolysis (as a side reaction or direct reversal);
- Water promotes agglomeration of catalyst particles, leading to mass transfer limitation of rates;
- Water may act as a competitive inhibitor for any of the substrates.

It is often difficult to prepare systems of reproducible water content just by adding a known amount. Water from other sources may be significant as well as that added deliberately. Water may be introduced associated with reactants, solvents or the biocatalyst preparation. It can also enter from (or escape to) the environment. Furthermore, if the effects of other parameters are being studied, experiments at

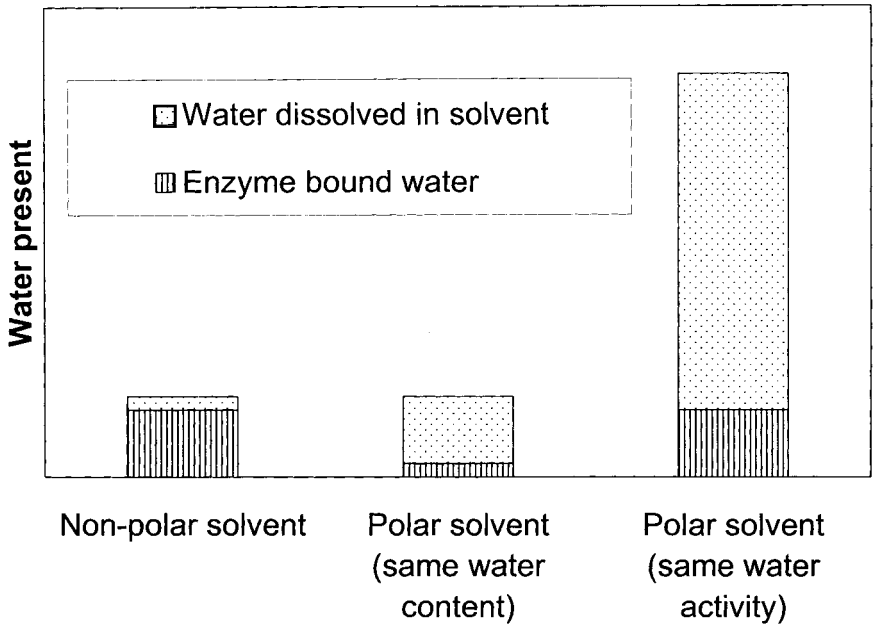

Figure 8-2. Quantities of water present in different phases when solvents are compared at equal total water content or equal water activity. The behavior of the enzyme is likely to depend only on the amount of water bound to it.

fixed water content can lead to very misleading conclusions. The water present will end up distributed between several different phases in the reaction mixture. Some will be closely associated with the enzyme molecules, and it is this quantity that will mainly affect their behavior. But some water will be dissolved in the bulk phase of the reaction mixture (e.g. the organic liquid). More will be associated with other solid phases present, such as an immobilization support and some may be present as vapor in a gaseous headspace. Changing these other phases (e.g. changing the nature of the organic solvent or the immobilization support) will affect the amount of water they retain, and hence that available to the enzyme. So what may appear to be an effect of solvent etc. may in fact be an effect of water. Put another way, the optimal water content (on a mass or volume basis) will change when alterations are made in several other factors. This is illustrated in Fig. 8-2.

It is increasingly accepted that a better parameter to characterize the water levels in these systems is its thermodynamic activity. This is defined as 1 in pure water, and will take on lower values in the various reaction media. Water will tend to transfer between the various phases present until they all reach equal water activity. Hence the whole reaction mixture will tend to come to a single equilibrium value of water activity. This will reflect the amount of water in each phase. In particular, a given water activity will tend to correspond to a particular quantity of water associated with the enzyme molecules. Hence their behavior will be most simply related to water

activity of the system, and the relationship will often stay the same as other factors are changed (e.g. the solvent).

Even in terms of water activity, hydration effects are not quite so simple however. As well as the current value, enzyme behavior depends on the history of hydration to which the catalyst has been exposed. In other words, there can be strong hysteresis effects. Nevertheless, water activity values are usually the best basis to define the previous history reproducibly.

8.3.1
Fixing Initial Water Activity of Reaction Components

The reaction mixture for biocatalysis will be prepared by combining several components. To ensure defined water conditions in the final mixture, all these components should preferably be brought to known water activity beforehand. (It may be safe to disregard this for a component that has a limited affinity for water and makes up only a small proportion of the final mixture.)

Often the easiest way to set the initial water activity of components of the reaction mixture is by pre-equilibration with a saturated salt solution. The relative humidity or water activity is fixed above a saturated solution of a given salt at a known temperature. As water equilibrates in or out of the solution, solid salt will tend to dissolve or crystallize to maintain saturation and hence the fixed water activity in the headspace. Any other material placed in contact with the headspace will eventually equilibrate to the same water activity. The reaction mixture component can simply be placed inside a closed vessel together with the salt solution, such that water can transfer between the two via the vapour phase. Wide-mouth screw cap jars are convenient, with salt solution over the base and an open vial containing the sample (Fig. 8-3). The rate of equilibration depends on the surface areas exposed and the amount of water that must be transferred. Typically 1–2 days is sufficient for either solid biocatalyst preparations or liquid phases based on relatively non-polar organic solvents. The rate of equilibration may be checked by weighing or Karl Fischer analysis respectively.

Table 8-2 shows the water activity values generated by a selection of salts we commonly use, taken from the best literature source. Most of these values are weakly temperature dependent. However, it is essential to use them at controlled tem-

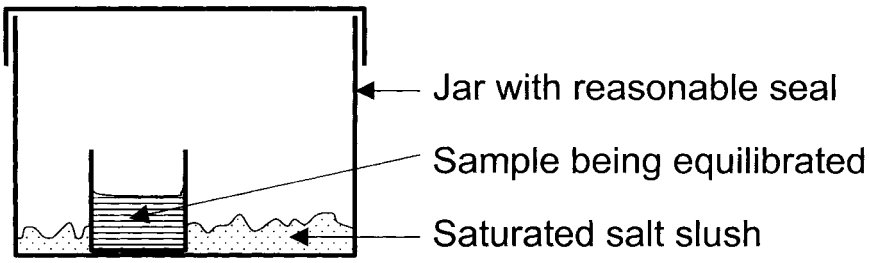

Figure 8-3. Method of pre-equilibration of water activity of reaction mixture components.

Table 8-2. Saturated salt water activities at 25 °C (from [18]).

LiCl	0.113	KI	0.689
KAc	0.225	NaCl	0.753
$MgCl_2$	0.328	KCl	0.843
K_2CO_3	0.432	KNO_3	0.936
$Mg(NO_3)_2$	0.529	K_2SO_4	0.973
NaBr	0.576		

perature, as fluctuations can cause the liquid phase to move away from saturation. The saturated solution is best prepared to have a lot of crystals surrounded only by thin layers of liquid, which will then re-equilibrate with the solid more quickly. Good sample purity is important when such large amounts of solid are used.

For water activities below 0.05, drying agents are required rather than salt solutions. Agents suitable for exhaustive drying are described in many conventional reference sources. Molecular sieves are popular in applied biocatalysis, but I would note two cautions. Firstly, if they are reactivated by heating, about 350 °C is required to obtain maximal drying efficiency. Secondly, if placed in direct contact with a liquid phase, they can have significant acid-base effects.

With water-miscible solvents, the organic phase can be prepared at the desired water activity more conveniently by simple addition of water to the dry solvent. The water concentration required will be significant, and the amount of water added will be much larger than unintentional exchanges with the environment or residual water levels in the dried solvent. The relationship between water concentration and activity will be more or less fixed for a given solvent, and little affected by reasonably low concentrations of reactants. This will not be true for less polar solvents, where direct addition of water rarely gives reproducible hydration or water activity. Table 8-3 gives water contents of various solvents at different water activities.

A decision must be made about the sequence and timing in which components are combined to make the final reaction mixture. The choices made can have large effects on the final hydration conditions and biocatalyst behavior. It is usually best initially to prepare as separate phases: (i) a non-aqueous solution or mixture of the reactants; and (ii) the solid biocatalyst preparation (lyophilized powder, immobilised enzyme, cross-linked crystal etc). The best treatment to apply then depends on the objective of the experiment.

- If the aim is to make a fair comparison of the effect of other factors (e.g. different solvents), then it is desirable to produce reaction mixtures of defined water activity. For this purpose, it is best if the two phases mentioned are separately pre-equilibrated to the target water activity before eventually combining them to start the reaction. In principle it is possible for the water activity to change somewhat from the pre-equilibrated value as components redistribute between the two phases. However, in practice such changes are small if the two phases noted are chosen. Another option is to pre-equilibrate the biocatalyst particles suspended in a non-aqueous fluid, and to add one final reactant at time zero. This reactant should be one added at fairly low concentration to prevent significant changes in water activity. These two options are illustrated in Fig. 8-4.

Table 8-3. Water contents (v/v%) of various solvents equilibrated at different water activities.

Water activity	0.05	0.1	0.2	0.4	0.6	0.8	1
Ethanol	0.449	0.94	2.06	5.2	11.0	28.5	M
2-Propanol	0.280	0.58	1.26	3.06	6.0	12.2	M
tert-Butanol	0.166	0.34	0.75	1.86	3.7	8.2	M
Dioxane	0.172	0.36	0.82	2.25	5.8	27.3	M
Acetone	0.188	0.39	0.86	2.18	4.56	11.7	M
Acetonitrile	0.154	0.32	0.72	1.86	4.19	15.3	M
Tetrahydrofuran	0.089	0.185	0.402	0.98	1.94	4.02	M
Ethyl acetate	0.066*	0.137*	0.298	0.73	1.44	2.99	–
Methyl *iso*-Butyl ketone	0.0529*	0.1061*	0.214	0.43	0.66	0.89	1.12
Methyl *tert*-Butyl ether	0.0470*	0.0940*	0.189*	0.38	0.58	0.78	0.98
iso-Propyl ether	0.0178*	0.0356*	0.071*	0.143*	0.216	0.290	0.364
Toluene	0.0015*	0.0029*	0.0058*	0.0117*	0.0175*	0.0233*	0.0292*
Hexane	0.0004*	0.0007*	0.0015*	0.0029*	0.0044*	0.0058*	0.0073*

Water contents are given as conventionally in terms of the volumes of pure liquids mixed to reach the required composition. With water-immiscible solvents, a water activity close to 1 is achieved in the mutually saturated system (as shown), but for miscible solvents (M), water activity 1 means pure water! Water activity of water-miscible solvents are estimated using the correlations derived by Bell et al.[19]. For water-immiscible solvents, they are based on water solubility measurements[20,21], and the approximation of constant activity coefficient up to saturation.

* – It is not advised to try to obtain the water activities shown by adding these small amounts of water to dry water-immiscible solvents. The values are given purely for use in estimating water quantities present or required. Apart from usual errors, small water droplets can take a very long time to dissolve in such solvents. Attempting such a method with solvents like toluene or hexane is particularly disastrous.

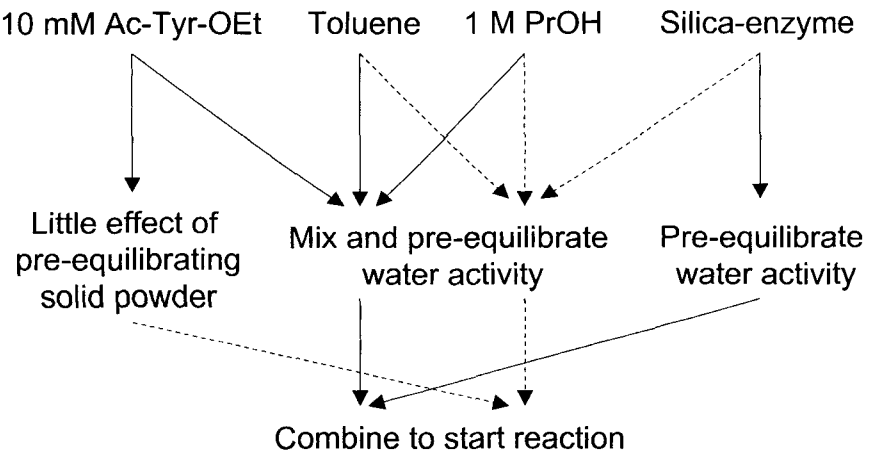

Figure 8-4. Choices in water activity pre-equilibration before a reaction in organic medium. The solid and broken arrows indicate two alternative schemes that can be considered. Other conceivable schemes will probably lead to a final reaction mixture of water activity very different from that used in pre-equilibration.

- On the other hand, if the aim is to achieve maximal catalytic activity in otherwise fixed conditions, it is often better to transfer the enzyme catalyst quickly and directly from an aqueous environment. Excess water can be removed if necessary by rinsing with a suitable polar solvent. It is best if the polar solvent contains enough water to bring it to the target water activity of the final reaction mixture. It is still best to ensure that the non-aqueous fluid phase starts with a defined hydration level, usually by pre-equilibration.

In either case, it is worth noting that achievement of a desired final water activity value is much easier in relatively polar solvents, where the substantial water concentration in the bulk phase will effectively buffer the whole system.

Two factors should be mentioned that can lead to significant unintended (and rather irreproducible) changes in water activity. Firstly, exchange of water with the environment. When only very small quantities of water are present (e.g. in media based on non-polar solvents), significant changes are possible. To avoid them, reaction vessels should be carefully sealed, and it may even be necessary to sample through a septum or a similar membrane. Sealing sufficient to prevent noticeable losses of volatile organic solvent may still allow significant water exchanges, because of the much smaller total quantities involved. Secondly, water may distill to any cold surface in contact with the headspace above a reaction mixture. This can lead to significant removal of water from the liquid phase, especially if the temperature difference is large. For example, a surface at 20 °C will condense water away from a reaction mixture at 60 °C until its water activity falls to 0.12 (the ratio of saturated vapor pressures). It is best to prevent this problem by eliminating all such cold spots. Reaction vessels may be surrounded by an air bath (taking account of explosion risk if flammable solvents escape), or completely immersed in a water bath. If individually jacketed vessels are used, unjacketed surfaces may be heated above the circulating water temperature.

8.3.2
Control of Water Activity During Reaction

Some methods offer the possibility of control of water activity during the reaction at the cost of greater complexity and/or the possibility of interference with the desired conversion. Most obviously a change in water activity may be due to water being produced or consumed in the reaction. However, it is also possible by water exchange with the environment (e.g. during sampling). With non-polar solvents water activity can also change because conversion of substrates to products will change the solvation of water. Hence water activity at a fixed concentration will be altered.

One simple and convenient method is the addition directly to the reaction mixture of suitable pairs of solid salt hydrates. A given salt hydrate will give up its water at a characteristic water activity, transforming to a lower hydrate or an anhydrous form. If the pair are placed in a system of water activity below their characteristic transition value, the (higher) hydrate will tend to give up water to the rest of the system. Water release will continue until the whole system reaches the transition water activity (or

the higher hydrate is completely exhausted). If on the other hand the salt pair are added to a system of high water activity, the form with less (or no) water will tend to take up water, transforming back to the (higher) hydrate. Once again, this will continue until the system water activity has been reduced to the transition value, or the salt form with less water has been completely consumed. In principle these exchanges should be able to buffer the water activity of the systems at the transition value of the added salt pair, provided enough is added. Typically, the higher hydrate will give up water at the start, as the reaction mixture is prepared from dry ingredients. Later, the lower hydrate may take up water produced in a reverse hydrolysis reaction. An example system is represented by the equation:

$$Na_2SO_4.10H_2O \text{ (crystals)} \rightleftharpoons Na_2SO_4 \text{ (crystals)} + 10H_2O \quad (1)$$

in which equilibrium with both solids present can only be reached at a single water activity value, 0.80 at 25 °C. Figure 8-5 illustrates the use of this hydrate pair in a synthetic reaction.

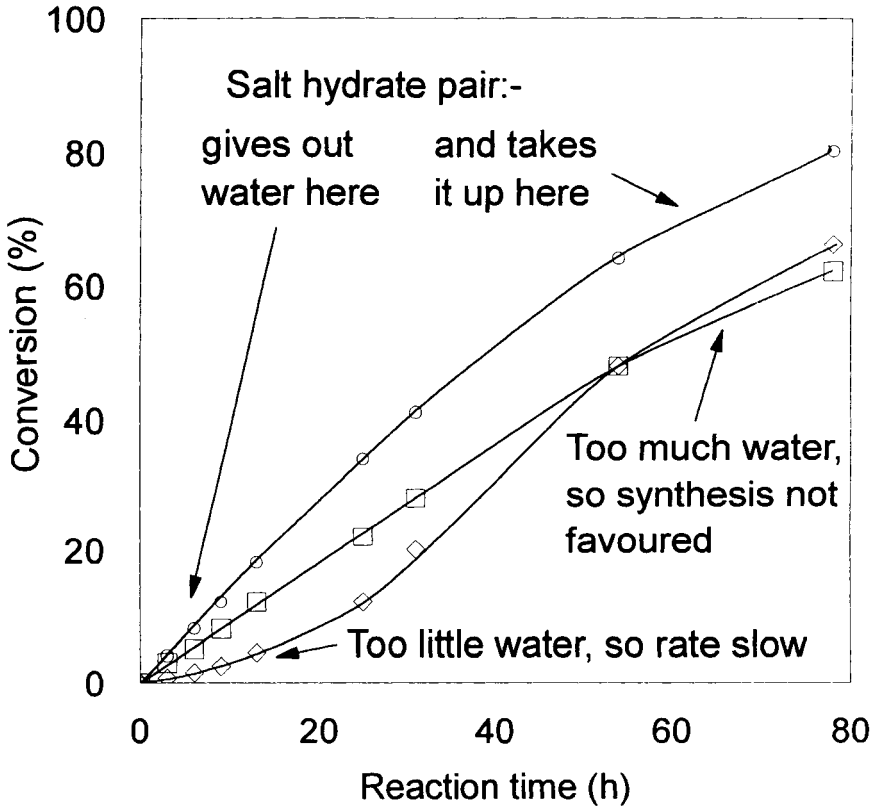

Figure 8-5. Control of water activity by adding salt hydrates to reaction mixture. Synthesis of butyl butanoate catalyzed by *Candida rugosa* lipase. Control reactions with catalyst relatively wet (□) or dry (◇) initially. Reaction in the presence of Na_2SO_4 plus $Na_2SO_4.10H_2O$ (O). Kvittingen et al.[22].

Table 8-4. Selected salt pairs found useful for water activity control in biocatalysis.

Salt pair	Equilibrium water activity	Rate of water transfer	Maximum temperature (°C)
NaI.2/0	0.12	fast	68
Na_2HPO_4.2/0	0.16	fast	95
Li_2SO_4.1/0	0.17	slow	233
NaAc.3/0	0.28	fast	c 58
NaBr.2/0	0.35	slow	50
$Na_2S_2O_3$.5/2	0.37	slow	48
$K_4Fe(CN)_6$.3/0	0.45	slow	87
$Na_4P_2O_7$.10/0	0.49	slow	c 80
$CaHPO_4$.2/0	0.50	slow	> 100
Na_2HPO_4.7/2	0.61	fast	48
Na_2HPO_4.12/7	0.80	fast	35
Na_2SO_4.10/0	0.80	fast	32

The pairs used are identified by a shorthand notation: NaI.2/0 means a combination of NaI.2H_2O and anhydrous NaI (i.e. 0H_2O). Equilibrium water activity values are for 25 °C. "Fast" water transfer indicates equilibration in a few minutes, "slow" that several hours may be needed. There is only limited information on the behavior of hydrate pairs giving lower water activities, though some indication that they generally tend to equilibrate slowly. From Zacharis et al.[23].

All of this describes just the thermodynamically favored directions of water transfer, for ideal crystalline solids. Many salt hydrate pairs seem to behave approximately ideally. However, if water activity is to be controlled close to the transition value, the rates of water release and uptake must be sufficient. Different salt pairs have very different rates of water exchange. It is difficult to give quantitative values, because the rates will depend on the size and shape of the crystals in each of the salt hydrate forms. This will depend on how they have been crystallized and handled subsequently. For example, cycling between hydrate forms, with gain and loss of water, will usually lead to a reduction in crystal size, and hence more rapid water exchange in future cycles.

The equilibrium water activity achieved depends on the choice of salt hydrate pair used and the temperature. In most cases the temperature dependence is higher than for saturated salt solutions. There is also a maximum temperature at which the higher hydrate will "melt" to give a liquid phase, so above this the biocatalyst will probably be seriously affected. Table 8-4 gives water activity values for some pairs that can be recommended for biocatalysis, together with an indication of the rates of transfer, and the maximum temperature. A compilation from the literature[24] gives information on temperature dependence, and notes some other hydrate pairs whose use has not been (fully) tested.

Many chemists have adopted the direct addition of salt hydrates as a simple method of water activity control. However, it does require a little thought and care to make sure the desired water activity is really produced. In particular, it must be ensured that both solid salt forms really will be present at equilibrium. It is best to estimate a "water budget" for the system, to ensure that enough of the right salt forms are being added. Table 8-5 shows an example of this, for a system made up of

Table 8-5. Example water budget for the use of salt hydrates.

Phase	Initial water content (μmol)	Estimated equilibrium water content (μmol)	Change (μmol)
10 mL toluene	55 (0.01% w/v)	128	+73
10 mg immobilised enzyme (on silica)	500	430	−70
100 mL gas headspace	26 (lab air, 20% RH)	104	+78
Water produced by esterification reaction from 10 mM substrates	–		+100

Water is assumed to show ideal dilute behaviour in toluene up to the solubility limit (16 mM). Equilibrium water content of immobilised enzyme estimated from measured adsorption isotherm (mainly adsorbed by silica support).

the phases shown, to be controlled at water activity 0.80 at 25 °C using the pair $Na_2HPO_4.12/7$. This example has been selected as one in which all four contributions are significant. More usually, one or two will dominate. In the example, the added salt hydrates will initially have to supply 73 + 78–70 = 81 μmol water to the reaction mixture. As the reaction proceeds, this will be all need to be taken up again, followed by another 19 μmol. Hence 81/5 = 16.2 μmol of $Na_2HPO_4.12H_2O$ should be able to supply the water required, transforming to the heptahydrate as it does so. To take up the last portion of the product water, 19/5 = 3.8 μmol of $Na_2HPO_4.7H_2O$ should also be added at the start. In practice, about 10 mg $Na_2HPO_4.12H_2O$ and 2 mg $Na_2HPO_4.7H_2O$ might be sensible, to ensure excess. Even more might be wise if the reaction vessel is likely to be opened frequently, allowing loss of water to the surrounding air.

In some cases, estimation of a water budget may indicate that buffering can be achieved by adding just one hydrate form, with the other formed *in situ* as water is given out or taken up. This is particularly attractive where one of the salt forms is not available. However, it is clearly wise to adopt this approach only when the direction of net water exchange with the reaction mixture has been very confidently determined. If not, the second hydrate form required can often be made fairly easily by hydration or drying of the one that is available.

Some limitations of the addition of salt hydrates must be borne in mind. In some cases the added salts may have additional, undesirable effects. They may react chemically with compounds involved in the enzymic reaction. For example $Na_2CO_3.10H_2O$ will neutralize carboxylic acids to their Na salts. More subtly, even quite weakly basic or acidic salts may exchange H+ with the enzyme molecules, affecting their behavior (see below).

There have been some cases of confusion in the control of water activity between saturated salt solutions (see above) and salt hydrate pairs. These can both be useful methods, but the principles and recommended applications are quite different. Avoid phrases like "control of water activity using salts", which do not make it clear which method is being used.

Water activity can be controlled during the reaction via the vapor phase, as in pre-

equilibration. Once again, saturated salt solutions are the best method of generating a vapor phase of controlled water activity. However, if the reaction produces or consumes water at significant rates, simple diffusion via the vapor phase will usually be too slow to maintain constant water activity. Forced circulation of the gas phase may give sufficient rates. For best water transfer, it can be bubbled through both the salt solution and the reaction mixture. There is an alternative method to achieve faster water exchange between a saturated salt solution and the reaction mixture. The two may be brought into contact across a membrane, so that only a very short diffusion path separates them (at the cost of a smaller diffusivity of water within the membrane). Microporous or ultrafiltration membranes may be best in principle, but for laboratory use one convenient solution is to use silicone tubing[25]. This is resistant to most organic solvents, and offers reasonable water permeability.

In some reactions the objective may be to remove water as vigorously as possible. This will lead to a low water activity, which would result in very poor catalytic activity of many enzymes. However, some enzymes are much more tolerant of low water activity. In this case, exhaustive dehydration may be the best policy, particularly to minimize hydrolysis reactions or maximize their reverse. In general, the methods adopted can be based on those used in conventional synthetic chemistry for handling water-sensitive materials. However, many of the most powerful drying agents cannot be used when they might come into contact with the enzyme, because of catalyst inactivation. For direct addition to the reaction mixture, the usual choice is molecular sieve. Type 4A is most commonly used, and is effective because nearly all the solvents have sufficiently large molecules that they are completely excluded. One piece of practical information is not as widely known as it should be. If molecular sieves are to be reactivated after use, very severe treatments are necessary to restore their full water-adsorbing power. If heating alone is used, a temperature of 350 °C is needed. It has recently become clear that molecular sieves can affect enzyme behaviour by acid-base effects as well as water removal. If the components of the reaction mixture are all relatively involatile (e.g. in solvent-free esterification), water removal by evaporation can be another effective method.

8.3.3
"Water Mimics"

One approach to improving catalytic activity at low water activity should be mentioned. Small additions of certain very polar liquids have been reported to greatly enhance catalytic activity at low water activity. They are usually described as "water mimics", and seem able to replace at least some of the roles of water in facilitating enzyme activity. Most of them are strongly hydrogen-bonded associated solvents that show other behavior analogous to water, such as glycerol, glycols and formamide. However, strong effects have also been observed with methanol and dimethylsulfoxide, for example. Most of the studies with these additives have been made with lyophilized powders, and hence may in part reflect the low control activities of these preparations (see Sect. 8.2). However, some significant effects have been reported with other enzyme forms, so I would recommend that use of such water mimics be

considered. They are clearly particularly attractive where very low water activity is desirable to prevent unwanted side reactions. Obviously the water mimic chosen must not promote analogous side reactions, such as with its hydroxyl groups.

8.4 Temperature

The pattern of temperature effects is the same as in aqueous media. The initial rate of reaction increases with temperature, in the usual Arrhenius fashion. However, the stability of the enzyme will decline with temperature, and at high enough values catalytic activity will be lost rapidly before significant conversions are reached. Hence, for given conditions, there will be an optimum temperature to maximize product yield after a given time. This is rarely a real fixed optimum for the enzyme, and for example will usually become higher if the reaction time is reduced. Progressive enzyme inactivation will have less effect over a shorter reaction time.

One important feature that can be exploited in low-water media is an increase in stability to temperature. Hence reactions may be carried out at temperatures higher than would be possible in aqueous media, often by many tens of degrees. It is fairly clear that the most important factor here is the amount of water in the molecular environment of the enzyme molecules, as determined primarily by the water activity of the system. The presence or nature of a solvent has little additional effect. Thus, beware of statements that "enzymes become more thermostable in organic solvents". It is the reduction in hydration that increases stability. If anything, the presence of an organic solvent will be destabilizing (in a comparison at equal water activity). In an organic solvent at water activity close to 1 (i.e. water saturated), the stability will be no better than in water. If, however, water activity is reduced to substantially below 1, a very valuable increase in stability may be achieved.

8.5 Substrate (Starting Material) Concentrations

Substrate concentrations affect catalytic rates in the same general way as in aqueous solution. At low substrate concentrations the rate is roughly proportional to [S] (i.e. first order kinetics). At higher concentrations the enzyme becomes saturated with substrate and the activity approaches a maximum limiting value. The full dependence is often described quite well by the Michaelis-Menten equation or its analogs for the more common two-substrate case (general two-substrate model, or the Ping-Pong model). These equations include a K_m parameter for each substrate, with units of concentration. When the actual substrate concentration is many times larger than its K_m value, the enzyme will be saturated with that substrate. Further increase in its concentration will then have little effect on the rate of reaction.

When the medium is changed, the K_m values will change also. An important contribution to this change has nothing to do with the enzyme directly, but reflects

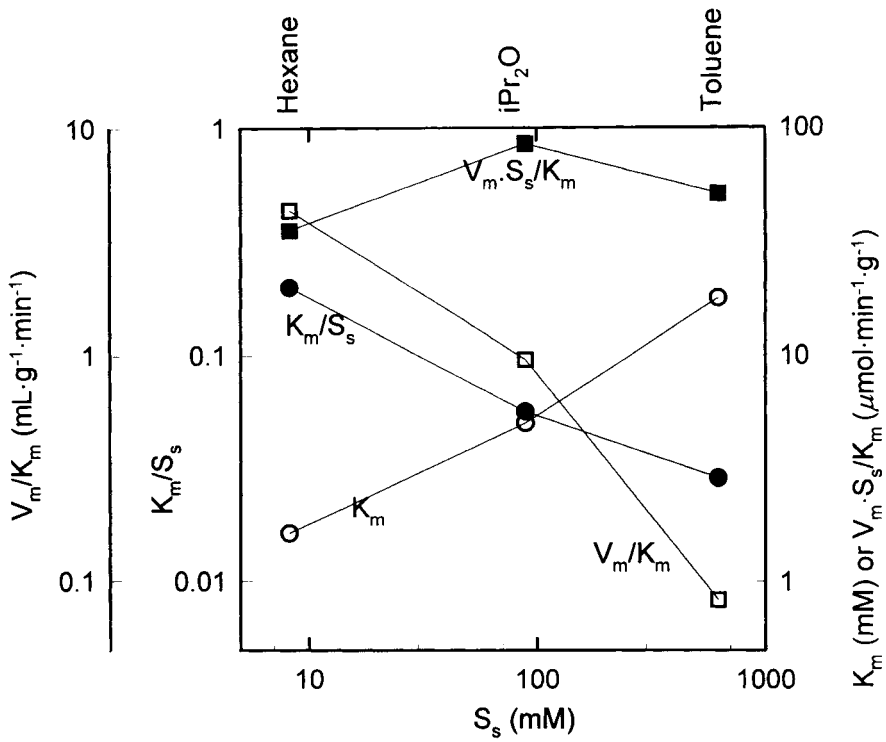

Figure 8-6. Kinetic parameters for subtilisin-catalyzed transesterification of Z-Ala-ONp in different solvents. Experimental Km (O) and Vm/Km (□) values are shown as a function of substrate solubility. The filled symbols show the corresponding "corrected" values, after allowing for substrate solvation. The variation in V_m/K_m is largely explained by solvation, while the "real" variation in K_m is opposite to the apparent trend. Reimann et al.[26].

the changed solvation of the substrates in the different media. Often this effect accounts entirely for the observed change. A simple quantitative picture is based on the relationship of K_m values to substrate solubility: the ratio of these will be approximately the same in each different medium. Figure 8-6 illustrates an example of this effect.

Often experiments to screen different solvents will keep the same substrate concentration in each. Hence, if a solvent in which the substrate is more soluble is tested, the K_m value will be increased, and the reaction rate may fall, as the enzyme is more limited by the availability of substrate.

For preparative syntheses, good general advice is to use a saturated solution of the substrate(s) in any solvent tested. This will only be a poor choice in the relatively rare cases of substrate inhibition. It will certainly be a good policy to allow identification of any direct effects of the solvent. An obvious way to ensure that the medium is saturated with substrates is to include excess in the form of solid particles. This leads

towards the mainly solid reaction mixtures mentioned elsewhere, and can be a good option in practice.

8.6
Solvent Choice

A large number of solvents might be chosen to form the basis of the low-water medium. The choice of solvent will usually have important effects on the rate and selectivity of the reaction, and on the stability of the biocatalyst.

8.6.1
Effects on Equilibrium Position

In many biocatalyzed reactions, the position of chemical equilibrium is important, because it will place a limit on the eventual yield. In such cases, the choice of solvent will usually have a significant effect on the equilibrium position. Because this simply reflects the differential solvation of reactants and products, these effects can be predicted fairly confidently, at least to a reasonable approximation [27].

One of the equilibria most commonly of interest is esterification. It may be desired to hydrolyze an ester, or reverse this in condensation of an alcohol and acid. Alternatively the hydrolytic equilibrium may be an undesirable side-reaction during transesterification. In this case, at a given water activity, the equilibrium position is quite strongly solvent dependent. The fraction of ester will increase dramatically on going from a polar solvent to non-polar solvent (Fig. 8-7). Hence alkanes are preferred solvents for esterification, while acetonitrile, a ketone or tertiary alcohol would be best for ester hydrolysis. If the equilibrium constant is expressed in terms of concentrations (including that of water), it is relatively solvent independent. However, optimal enzyme behavior in the different solvents usually requires maintaining the same water activity. At fixed water activity, the ratio of ester to acid and alcohol concentrations will be maximized in the least polar solvents.

8.6.2
"Solvent Effects" that Really are Not

Many apparent "solvent effects" reported in the literature are actually due to changes in the availability of water or substrate to the enzyme. It is commonly observed that activity appears to be highest in the least polar solvents. Sometimes the explanation will be added that these "have the least tendency to strip water from the enzyme". This undoubtedly indicates a common mechanism, but in such cases the "solvent effect" will disappear completely if experiments are run at equal water activity, as recommended in the discussion above (Sect. 8.3.1 and Fig. 8-2). Many other observed "solvent effects" operate via changes in substrate solvation, as explained in Sect. 8.5. Hence, they are really effects of changing substrate availability when different solvents are compared with equal substrate concentrations.

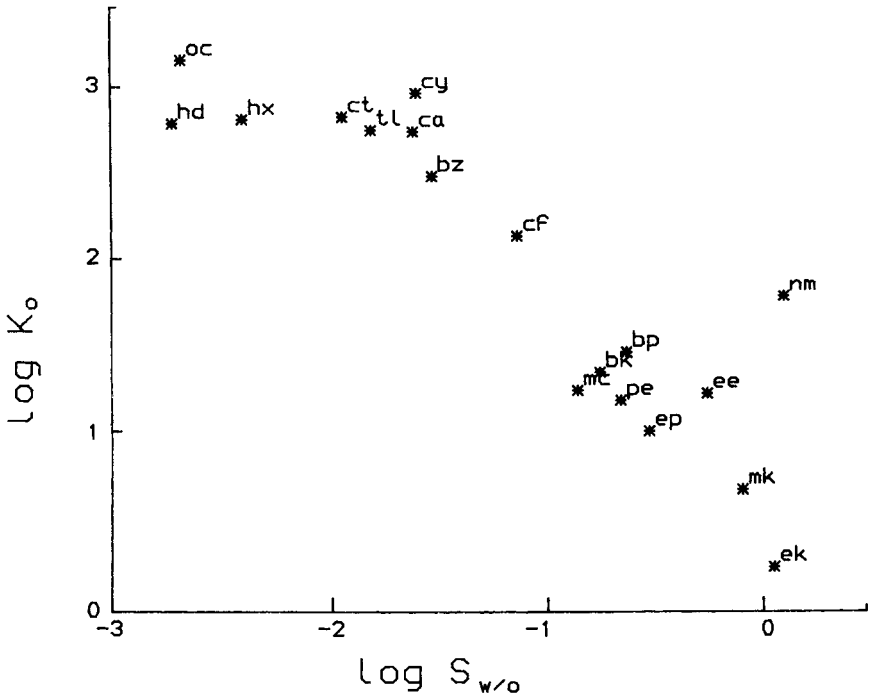

Figure 8-7. Correlation between equilibrium constant for esterification and solubility of water in the solvent. Equilibrium constant was defined as [Ester]/([Alcohol].[Acid]), for reactions at fixed water activity (close to 1). Solvents are: bb, butyl benzoate; be, bromoethane; bk, dibutyl ketone; bp, dibutyl phthalate; bz, benzene; ca, 1,1,1-trichloroethane; cf, chloroform; ct, carbon tetrachloride; cy, trichloroethylene; ee, ethyl ether; ek, diethyl ketone; ep, diethyl phthalate; hd, hexadecane; hx, hexane; mc, methylene chloride; mk, methyl *iso*-butyl ketone; nm, nitromethane; oc, *iso*-octane; pe, *iso*-propyl ether; tl, toluene. Valivety et al.[28].

8.6.3
Solvent Polarity Trend and Recommended Choices

A very commonly observed trend is that activity is highest in the least polar solvents. In many of these cases this is an effect of water or substrate availability, as just noted. Hexane is regularly identified as the best medium, because the low solubility of water and most substrates makes them most available to the enzyme, when comparisons are made at equal concentrations. Nevertheless, even when water and substrate availability have been allowed for, non-polar solvents seem to offer the highest activity. The probable explanation involves the tendency for solvent molecules to migrate from the bulk phase into the immediate environment of the enzyme. The picture is simplest when there is a discrete aqueous phase (albeit of very small volume) around the enzyme molecules. The more hydrophobic the bulk solvent, the lower will be the (saturating) concentration in the aqueous phase, which is what is experienced by the enzyme. Even in the absence of an identifiable aqueous

phase, the immediate environment of the enzyme molecules will be more polar than the bulk.

Hence, it is often best to select a non-polar or hydrophobic solvent, at least for initial trials. Some reasons why this might not be the best choice are:

- If the reaction wanted is a hydrolysis, the equilibrium will be less favourable than in a polar solvent (see above);
- The reactants may be only poorly soluble; however, using a suspension of incompletely dissolved substrates may still be a good policy. Provided the rate of dissolution and concentration in solution are sufficient, a good reaction rate can still be achieved.

The following list presents some choices for a more general solvent screening exercise:

- An alkane: *n*-hexane is most commonly used, although on safety grounds cyclohexane, heptane or isooctane would be preferred.
- An aromatic hydrocarbon: toluene would usually be preferred over benzene on safety grounds.
- An ether: diethyl ether is usually inconveniently volatile, and popular alternatives are di-*iso*-propyl ether or methyl *tert*-butyl ether.
- A ketone: methyl *iso*-butyl ketone or acetone. Being miscible, the latter may not be suitable if a medium of high water activity is required – this will end up as a high water content mixture that may dissolve and denature the enzyme.
- A tertiary alcohol: *tert*-pentanol or *tert*-butanol. These are useful because they do not react with most enzymes that accept alcohol substrates.
- A water-miscible but aprotic solvent: one of tetrahydrofuran, dioxane or acetonitrile.
- A small alcohol. Either ethanol or 2-propanol is probably best. These solvents must be avoided for many enzymes, as they will be reactive, for example as nucleophiles or reductants. Methanol as a pure solvent is often particularly inactivating.
- Chlorinated solvents can have some distinctive properties but are usually avoided for two reasons. On safety and environmental grounds, they are increasingly disfavored for large scale applications. They also tend to be more inactivating to biocatalysts than other solvents of similar polarity. (In some cases this may in fact be due to the stabilizers added to most chlorinated solvents.)

Supercritical fluids have advantages as reaction media for large scale applications, but the need for high pressure apparatus means they will not usually be favoured for laboratory syntheses. Volatile reactants can be supplied to a catalyst through a gas phase, and the higher temperature stability under low water conditions makes this applicable to more cases than might first be thought. However, the increased complexity of apparatus again makes this more likely to be favored only at an industrial scale.

8.6.4
Solvent Parameters

For preparative purposes, the idea of correlation with some qualitative idea of solvent polarity is often sufficient, as implied here. There are numerous parameters which can be used to quantify the difference between solvents, but they all show some correlation with each other. By almost any measure, we would obtain the order: hexane, toluene, methyl *iso*-butyl ketone, propanol. However, different parameters can give different rankings when more similar solvents are compared. For biocatalysis in non-aqueous media, there are few effects where the "correct" solvent scale can be confidently identified. However, it is useful to have an idea of two quite different classes of solvent scale.

- Most of them describe features of how the bulk solvent behaves and is able to interact with isolated solute molecules. These will be based on measurements on or in the solvent as a bulk medium. Different parameters measure different features of the interaction the solvent may have with solutes, e.g. dielectric, cohesiveness, acidity, basicity. When the behavior of the solvent as a bulk medium is being considered, it is appropriate to use scales from this group.
- In contrast, some parameters are properties of individual solvent molecules. Examples are dipole moment and log *P* (the octanol-water partition coefficient). These parameters are appropriate where individual solvent molecules are engaged in interactions away from the bulk phase. Thus, log *P* is used sensibly to describe the tendency of solvents to interact with (and affect the functioning of) the enzyme molecules. However, these parameters are not good choices when bulk solvent behavior is important, such as its ability to solvate water or reactants (and hence affect their availability to the enzyme). Even when such mechanisms are important, it is quite common to see correlations presented against log *P*. However, any relationship probably reflects the correlation of log *P* with appropriate scales of bulk solvent behaviour.

There is a tendency to use two different words that make a related distinction between different types of solvent parameter. The log *P* parameter can be called a measure of solvent "hydrophobicity", which is an accurate description of what affects its value. This contrasts with other parameters such as dielectric, which measure the bulk "polarity". One illustration of the difference is to consider homologous series of solvents. Adding extra methylene groups to an alcohol, for example, will cause a regular increase in hydrophobicity. The effect on polarity will be much less, however, as the hydroxyl groups can still be oriented to solvate a polar solute. Thus decanol is more hydrophobic (higher log *P*) than hexane, but will be more polar by almost any measure of bulk properties.

One illustration of the difference between these two classes of measure comes in the treatment of mixed solvents. For parameters that relate to the ability of the solvent in bulk to interact with solutes, it is meaningful to define and measure a value for a mixture of solvents. Often this will be a simple function of the mole or volume fractions and the pure solvent values. However, for parameters that describe

the behavior of individual molecules of the solvent, a value for the mixture is meaningless. The two types of solvent molecule present will behave differently and essentially independently.

8.6.5
Solvent Effects on Selectivity

Solvent effects on enzyme selectivity or specificity are very important. One of the attractions of non-aqueous media is the ability to tune these key properties, and substantial effects can certainly be observed. Unfortunately, it is not yet possible to give confident predictions in most cases.

Predictions can be offered for the effect on selectivity between two substrates. A major contribution here comes from differential solvation, and the selectivity at a fixed concentration ratio will depend on relative solubilities, as noted in Sect. 8.5. However, these effects are rarely of preparative relevance, as it is not common to use two competing substrates that differ greatly in solvation. Selectivity between enantiomers is often desired, but here solvation effects will not distinguish the two substrates (unless a chiral solvent is used). Changing the solvent can have important effects on the selectivity between enantiomers (up to 2 orders of magnitude, with inversion of stereopreference possible). The effects must by definition be based on differential solvent interaction with the two diastereoisomeric transition states. A model based on solvent interaction with exposed portions of the substrate moeities in these transition states can sometimes make correct predictions of the direction of the effect, although its generality needs more testing.

8.6.6
No Solvent or Little Solvent Systems

In many cases an attractive option is to use no "solvent" at all. In some cases at least one of the reactants will be liquid, so can be the basis of a fluid phase for transfer of reactants. If slightly raised temperatures are used, this condition will be met more often. (Remember that at reduced water activity, the enzyme will usually be stable to higher temperatures than in aqueous solution.)

Another option is to abandon the usual idea that most or all of the starting materials should be dissolved in order to get effective reaction. Attractive reaction mixtures can be prepared containing mainly undissolved solid particles of substrates. The reaction actually takes place in a liquid phase containing the enzyme, but this can be totally hidden between the reactant particles. The system formed is illustrated in Fig 8-8. Usually the liquid phase will be generated by adding a small amount (e. g. 10 % by weight) of a "solvent". Often the best solvent is water itself, as it will usually give the highest catalytic activity. In these mainly solid systems, this may be combined with many of the advantages of non-aqueous media, notably the reversal of the equilibria of hydrolytic reactions.

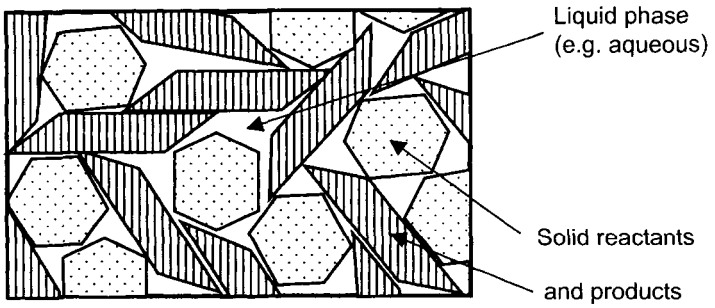

Figure 8-8. Schematic illustration of mainly solid reaction system. Starting material crystals will progressively dissolve, while product crystals will grow, as the enzymic reaction happens in the liquid regions between them.

8.7 Acid-Base Conditions

8.7.1 pH Memory

It is well known that pH has a major influence on the behavior of enzymes in aqueous media. Most who use enzymes under low-water conditions are aware of the phenomenon known as "pH memory". The activity and other properties of the enzyme are affected by the pH of the last aqueous phase to which it was exposed before drying for use in low-water conditions. This phenomenon is usually attributed to the relative rigidity of enzyme molecules at low hydration, by analogy with the effects of co-drying with additives. (see Sect. 8.2.5)

However, another picture may give a clearer view of pH memory and when it may prove insufficient to apply controlled acid-base conditions. In aqueous solution, pH influences enzymes by affecting the protonation state of acidic and basic groups in the molecule. At a given pH, the protein molecule will have a characteristic net charge. Electroneutrality requires that the surrounding solution contain an excess of oppositely charged counter-ions precisely to balance the protein charge. In aqueous solution these counter-ions are relatively far away, and their presence and identity has only limited effects on behavior. However, consider drying this portion of aqueous medium containing the enzyme. In general, the counter-ions present will remain, as only water is removed. So the net charge on the counter-ions, and hence the opposite net charge on the protein will be preserved. The requirement for electroneutrality means that the only possible changes in protonation state are internal H^+ exchanges between groups in the protein. Each such exchange will create or destroy a pair of positive and negative groups, without altering the net charge. In summary, this picture shows that pH memory resides in the behavior of the counter-ions as much as the protein, and does not require any special rigidity of the latter. This is illustrated in Fig. 8-9.

Also it should now be clear that pH memory is not a phenomenon unique to non-

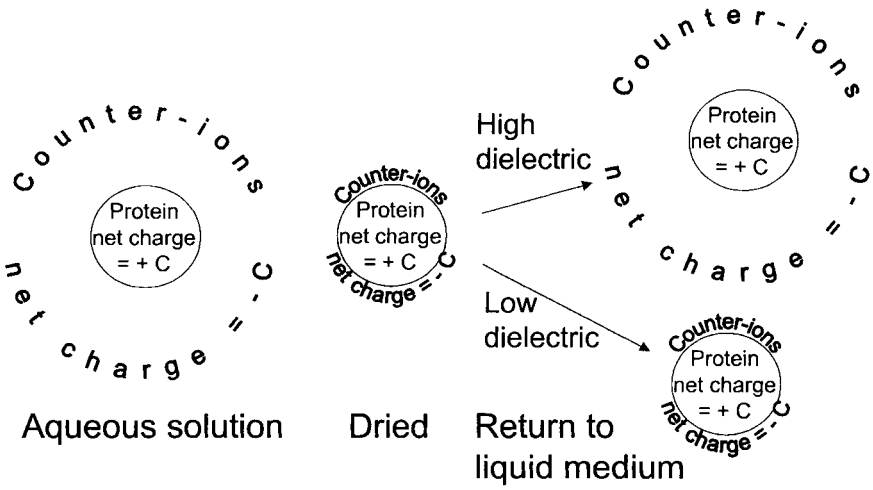

Figure 8-9. Illustration of the relationship between protein net charge and that on the counter-ions, and how drying and re-suspension or dissolution cannot change it.

aqueous media. If a dried enzyme preparation is placed back into pure water, its behavior will be determined by the pH value before drying – the ionization state of the protein, and the counter-ions present will effectively buffer the water back to the original pH value. Of course, we would not normally think of doing an experiment like this in aqueous media. The pH value reached would be very weakly buffered, and might be greatly altered by traces of acid or base from impurities, reactants etc. In low-water media, it is more common that acid-base conditions will not be seriously affected by these unintended effects. Hence pH memory may be sufficient to control behavior. However, there are several common ways in which pH memory may fail, which at least should be carefully considered before deciding to rely on pH memory. In addition, there are now established some relatively simple methods to buffer acid-base conditions in some low-water media, making reliance on pH memory unnecessary.

8.7.2
Processes Erasing pH Memory

As the picture presented above suggests, the net charge on the protein may be affected by processes leading to preferential loss of counter-ions of one charge. This can happen if counter-ions undergo proton exchange reactions with the protein to produce a neutral species. The exchange may be driven to completion if the neutral species produced is then removed from the neighborhood of the protein. Such exchanges may be relatively easy if the counter-ions are derived from weak acids or bases. If the acid or base is then volatile, the counter-ions can be lost during drying under vacuum, with changes in protein net charge, as represented by reactions such as:

$$\text{Protein-NH}_3^+.^-\text{OOCH} \rightarrow \text{Protein-NH}_2.\text{HOOCH} \rightarrow \text{Protein-NH}_2 + \text{HOOCH (gas)} \quad (2)$$

$$\text{Protein-COO}^-.\text{NH}_4^+ \rightarrow \text{Protein-COOH.NH}_3 \rightarrow \text{Protein-COOH} + \text{NH}_3\text{ (gas)} \quad (3)$$

A similar process can occur if the acid or base can be extracted into the bulk phase of the reaction mixture (e.g. octanoic acid or triethylamine in an organic solvent).

Other counter-ions may be exchanged with the bulk non-polar phase, provided something is able to solubilize them there. This will usually be in the form of an ion-pair with a species better solvated by the medium. For example, an acid with a large hydrophobic group may form a Na^+ salt with sufficient solubility in the bulk medium. The protonated acid will carry H^+ to and from the enzyme in exchange, to maintain electroneutrality. A similar process with a hydrophobic amine, for example, can transfer H^+ and Cl^-. Solubilization of the small ion may be aided by complexation, for example of Na^+ by a crown ether. The exchanges can be written as:

$$\text{Protein-COO}^-.\text{Na}^+ + \text{RCOOH (bulk phase)} \rightleftharpoons \text{Protein-COOH} + \text{RCOO}^-.\text{Na}^+\text{ (bulk phase)} \quad (4)$$

$$\text{Protein-NH}_3^+.\text{Cl}^- + \text{R}_3\text{N (bulk phase)} \rightleftharpoons \text{Protein-NH}_2 + \text{R}_3\text{NH}^+.\text{Cl}^-\text{ (bulk phase)} \quad (5)$$

Acidic or basic species in the bulk phase may protonate or deprotonate the enzyme, becoming the necessary counter-ions in the process. So we might have equilibria such as:

$$\text{Protein-COOH} + \text{CH}_3\text{NH}_2\text{ (bulk phase)} \rightleftharpoons \text{Protein-COO}^-.^+\text{NH}_3\text{CH}_3 \quad (6)$$

$$\text{Protein-NH}_2 + \text{CH}_3\text{COOH (bulk phase)} \rightleftharpoons \text{Protein-NH}_3^+.\ ^-\text{OOCCH}_3 \quad (7)$$

The protonation state of the enzyme may be affected by acidic or basic reactants (starting materials or products). These species could act as described by either of the two sets of equilibria just presented. Acidic or basic impurities in solvents could also be significant here.

8.7.3
Systems for Acid-Base Buffering

It should be clear that there are several possible mechanisms by which the protonation state of an enzyme may be altered in low-water media. It will often be desirable to try to maintain the optimal state by controlling acid-base conditions, rather than just relying on pH memory. This can be done by the addition to the reaction system of acid-base buffers, as in aqueous media. However, the details of these buffer systems and how they work is usually somewhat different.

The equilibria represented by Eqs. (4) and (5) can be employed to set up buffering based on agents dissolved in the bulk non-aqueous phase. As the equilibria indicate, the state of ionizable groups in the enzyme will depend on the ratio of buffer forms added to the bulk phase: the acid and its ion-paired salt with Na^+ (or another cation); the base and its ion-paired hydrochloride salt (or similar). Also in analogy to aqueous buffers, a given pair will only be usable over a given range of acidity/basicity. The

conditions where optimal buffering is found (analogous to aqueous p*K*) will depend on the solvent used. A number of such organic soluble buffer pairs have now been identified[29–31].

Identification of buffers that can be dissolved in the bulk phase is restricted by solubility, usually of the ionized form. An alternative approach is to choose buffers expected to be almost completely insoluble in the reaction medium, which will remain as suspended crystals. Convenient choices are zwitterionic solids and their salts, which will give rise to equilibria as shown in Eqs. (8) and (9).

$$\text{Protein-COO}^-.\text{Na}^+ + \text{TES}^{+-}\ \text{(crystals, zwitterionic)} \rightleftharpoons \text{Protein-COOH} + \text{TES}^-.\text{Na}^+\ \text{(crystals)} \tag{8}$$

$$\text{Protein-NH}_3^+.\text{Cl}^- + \text{Lys}^{+-}\ \text{(crystals, zwitterionic)} \rightleftharpoons \text{Protein-NH}_2 + \text{Lys}^+.\text{Cl}^-\ \text{(crystals)} \tag{9}$$

Since the buffer compounds are now present as crystalline solids, the equilibrium position is independent of the quantity of each. A given pair sets a characteristic protonation state of the enzyme. This is analogous to the use of solid salt hydrate pairs to set a hydration state. Again, to cover the range of acid-base conditions that might be appropriate for different enzymic syntheses, a series of different buffer pairs is required. A number have been identified[32–34], but the known range probably needs extending.

Of course, if such equilibria are to be established, a mechanism is required for the transfer of H^+ and counter-ions between the solid buffers and the enzyme molecules. Quite surprisingly, this usually does not seem to be a limitation. Only quite small quantities of ions must be exchanged, which will make equilibration easier. Probably traces of acids and bases soluble in the bulk phase can catalyze the transfers by equilibria such as Eqs. (4) and (5). If rates of equilibration are inadequate, deliberate addition of such transfer agents should help.

Although the analogy to aqueous acid-base behavior is clear, there are important differences. In particular, the ionization of acidic and basic groups in the protein becomes to a considerable extent independent. Both are affected by the availability of counter-ions as well as of H^+, as illustrated in equilibria like those shown in Eqs. (4) and (5). Hence in principle two different buffering systems should be used to fix the state of these two categories of protein groups. In a medium that is saturated with a simple salt (typically NaCl), these two different acid-base parameters become linked in a fixed relationship. In this case, the system reverts to having only a single acid-base variable, as in water. Only limited studies have been made so far of systems in which both classes of buffer are present, so it is not possible to say how often better performance can be obtained by optimizing both. Hence for the present, I would not advise those persons looking at practical syntheses to use more than one type of buffer.

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9
Enzymatic Kinetic Resolution

Jonathan M. J. Williams, Rebecca J. Parker, and Claudia Neri

9.1
Introduction

Conventional kinetic resolution procedures often provide an effective route for the preparation of enantiomerically enriched compounds. However, a resolution of two enantiomers will only provide a maximum of 50 % yield of the enantiomerically pure material. This limitation can be overcome in a number of ways, including inversion of the stereochemistry of the unwanted enantiomer, racemization and recycling of the unwanted enantiomer or dynamic kinetic resolution.

A dynamic kinetic resolution reaction involves the interconversion of the enantiomers of a starting material under conditions where one enantiomer is converted selectively into product. This principle is shown in Fig. 9-1, where a conventional kinetic resolution reaction and a dynamic kinetic resolution reaction are compared. In both cases enantiomer **A** reacts to form product **B** more quickly than enantiomer **A'**. However, in the conventional kinetic resolution, enantiomer **A'** is simply left behind as unreacted starting material. In the dynamic kinetic resolution, **A** and **A'** are in equilibrium, which allows for the possibility that all of the starting material will be converted into product **B**. The reaction conditions must be chosen that whilst the starting material enantiomers (**A**/**A'**) undergo rapid equilibration (racemization), the product **B** must be inert to racemization.

Dynamic kinetic resolution reactions are not limited to enzyme-catalyzed processes, and there are reviews available that consider all aspects of such reactions [1–3].

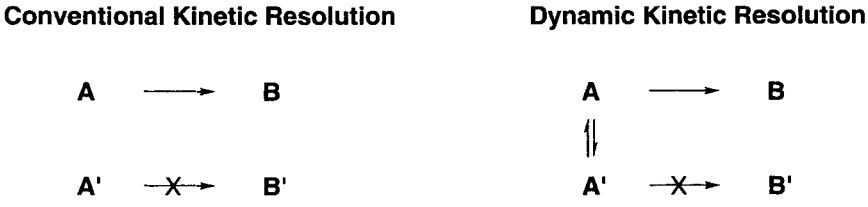


Figure 9-1. Comparison of conventional and dynamic resolution reactions.

In addition, reviews dealing with aspects of enzyme-catalyzed dynamic resolution and related processes such as stereoinversion and deracemisation have also been published[4–7]. Details of the kinetic principles of dynamic kinetic resolution reactions have also been reported[7–9]. Interestingly, a dynamic kinetic resolution reaction can provide a product with higher enantiomeric excess than the corresponding kinetic resolution. In a conventional kinetic resolution, the enantiomeric excess of the product often decreases as a function of conversion. This happens because as the reaction proceeds, the proportion of the preferred enantiomer of substrate decreases. Unless the enzyme is able to discriminate perfectly between the substrate enantiomers, it will catalyze the reaction of the less preferred enantiomer of substrate (the proportion of which grows as the reaction proceeds). However, in a dynamic kinetic resolution where the substrate enantiomers are interconverting rapidly, the ratio of substrate enantiomers will be constant at 1:1. Consequently, the enantiomeric excess of the product will not decrease as the reaction proceeds.

The following sections consider dynamic resolution reactions of alcohols (and their derived esters), acids (and their derived esters) as well as dynamic resolution involving reaction catalyzed by dehydrogenase enzymes.

9.2 Alcohols and their Derivatives

In order to achieve a dynamic kinetic resolution of alcohols, procedures need to be found for the *in situ* racemization of these substrates. The racemization conditions need to be compatible with the enzyme-catalyzed step, and the product must be inert to racemization.

The general principles are identified in Fig. 9-2, where enzyme-catalyzed acylation selectively converts one of the equilibrating alcohols into the corresponding ester.

Methods for racemization of the alcohol include substrates where the R or R' group is a good leaving group, or where temporary dehydrogenation to the corresponding ketone can be achieved, as shown in Fig. 9-3.

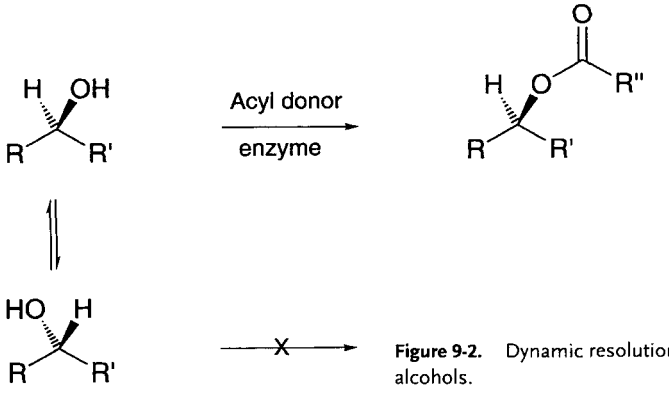


Figure 9-2. Dynamic resolution in the acylation of alcohols.

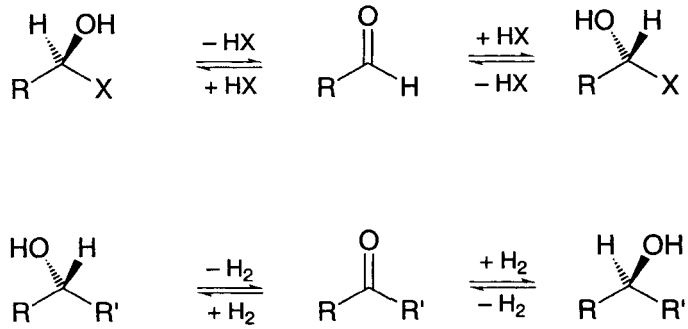
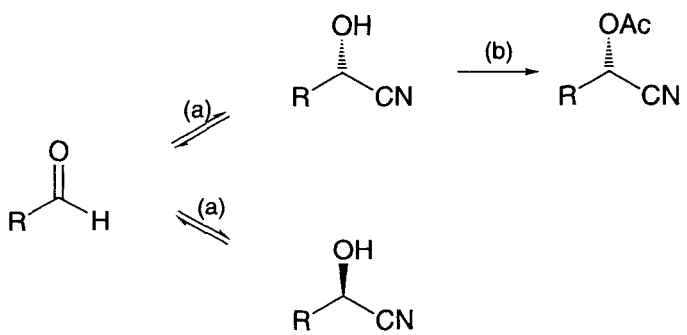


Figure 9-3. Racemization of alcohols via carbonyl compounds.

9.2.1
Cyanohydrins

Cyanohydrins are readily racemized with base, and this has been exploited by Oda and co-workers in a dynamic kinetic resolution of these substrates[10, 11]. In a typical procedure (Fig. 9-4), the cyanohydrins were formed by transhydrocyanation with acetone cyanohydrin, catalyzed by the hydroxide form of an anion exchange resin (Amberlite IRA-904). The reversible nature of the cyanohydrin formation allows racemization to proceed during the course of the enzyme-catalyzed acetylation, and the choice of isopropenyl acetate as the acyl donor means that the only by-product is acetone.

The immobilized lipase from *Pseudomonas cepacia* (Amano) afforded good enantioselectivities for the formation of a range of cyanohydrin acetates derived from aromatic aldehydes (Fig. 9-5). Polymer-supported quinidine could also be employed



(a) Acetone cyanohydrin, IRA-904 resin (HO^- form)
(b) *Pseudomonas cepacia* lipase, isopropenyl acetate
3Å molecular sieves, i-Pr_2O, 40 °C, 3-6 days

Figure 9-4. Racemization of cyanohydrins with *in situ* acylation.

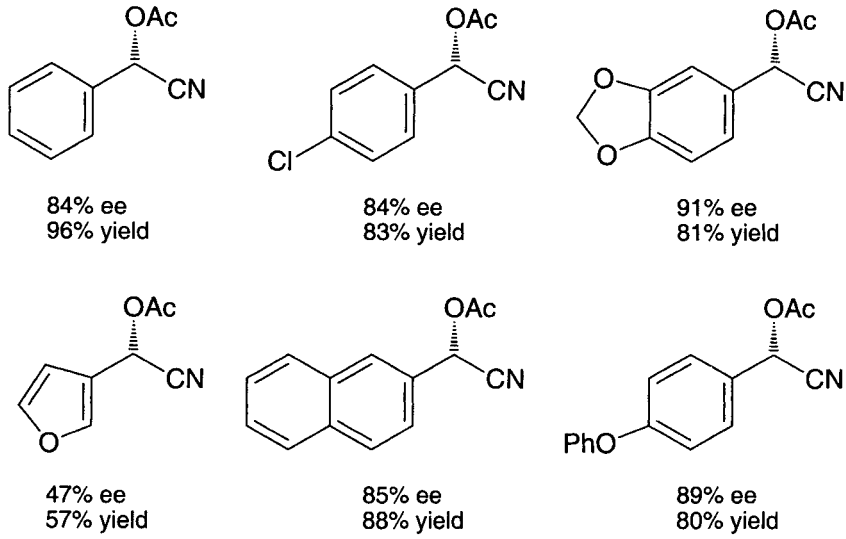


Figure 9-5. Examples of cyanohydrin acetates formed by dynamic resolution.

as the base for racemization and formation of cyanhydrins, although the reactions were generally slower than with the Amberlite resin [12].

9.2.2
Other Readily Racemized Substrates

Kellogg, Feringa and co-workers have achieved successful dynamic kinetic resolution reactions using cyclic hemiacetals as substrates [13, 14]. The enzyme-catalyzed acetylation of 6-hydroxypyranone shown in Fig. 9-6 has been achieved with reasonable enantioselectivity with essentially complete conversion. The racemisation of the hemiacetal is presumed to proceed via reversible ring-opening of the pyranone [13]. The rate of reaction was found to greatly increase when the enzyme, lipase PS (*Pseudomonas* sp.) was immobilized on Hyflo Super Cell (HSC).

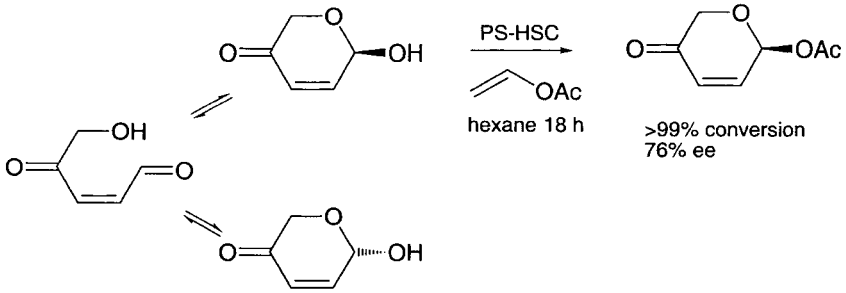


Figure 9-6. Dynamic resolution of cyclic hemiacetals.

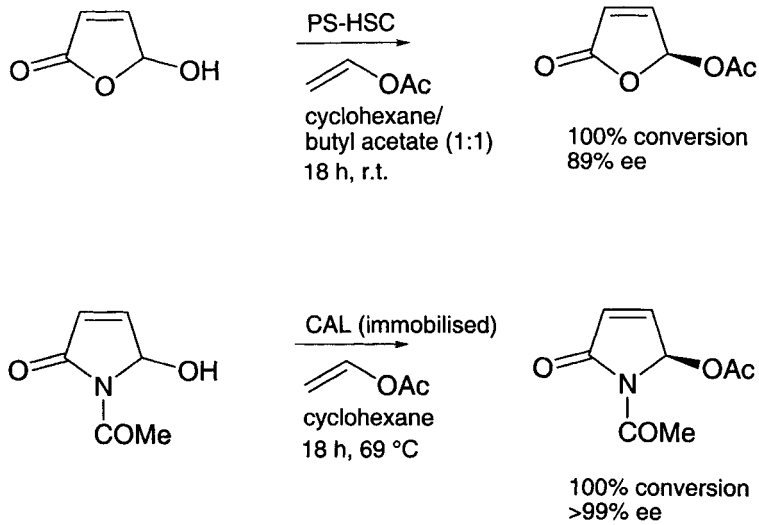


Figure 9-7. Examples of dynamic resolution of furanone and pyrrolinones.

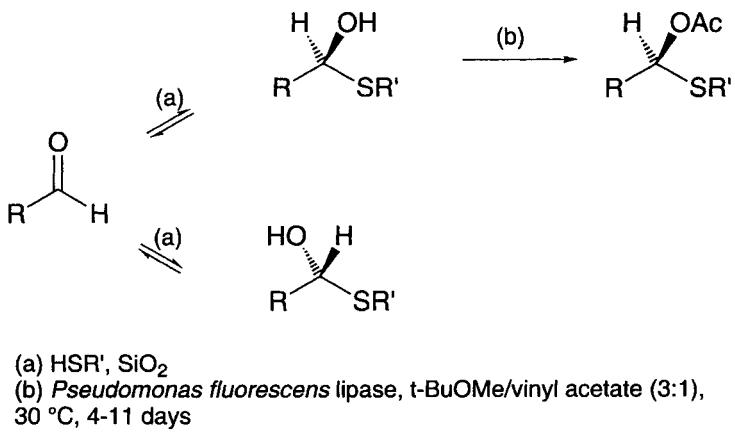


Figure 9-8. Dynamic resolution of hemithioacetals.

The related dynamic resolutions of the furanone and pyrrolinone substrates were achieved with higher selectivities (Fig. 9-7)[14]. Again, these substrates underwent spontaneous racemization under the reaction conditions. Appropriate choice of enzyme afforded a good example of an essentially perfect dynamic kinetic resolution process in the case of the esterification of the hydroxypyrrolinone substrate.

Rayner and co-workers have demonstrated that hemithioacetals can be racemized on exposure to silica[15]. In a typical experiment, an aldehyde and a thiol are combined to give a hemithioacetal. In the presence of silica, the enzyme-catalyzed acetylation proceeds under dynamic resolution conditions, as shown in Fig. 9-8.

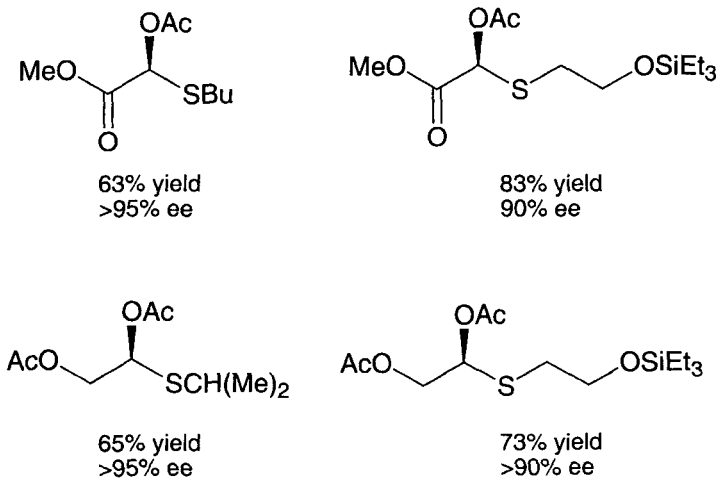


Figure 9-9. Examples of acetylated hemithioacetals.

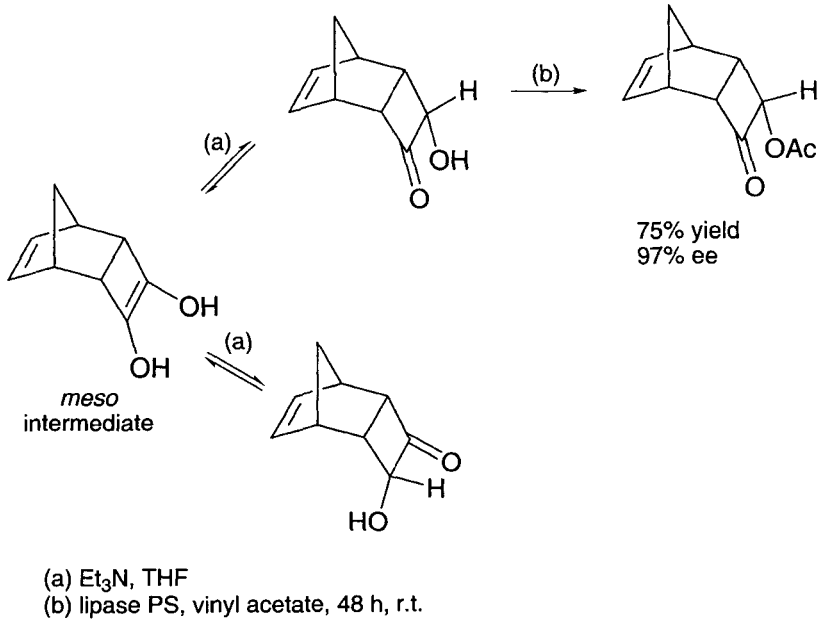


Figure 9-10. Dynamic resolution involving a *meso* intermediate.

Representative products obtained using this procedure are given in Fig. 9-9. The acetylated products are inert to racemization under the reaction conditions.

An interesting example of dynamic kinetic resolution of an alcohol has been reported by Taniguchi and Ogasawara[16]. The α-hydroxy ketone in Fig. 9-10 under-

goes racemization via a *meso* enediol intermediate, and one of the enantiomers can be acetylated selectively with lipase PS (*Pseudomonas* sp. immobilized on Celite, Amano). The added triethylamine was required in order for the racemization to take place. Without triethylamine, the reaction proceeded under conventional kinetic resolution conditions.

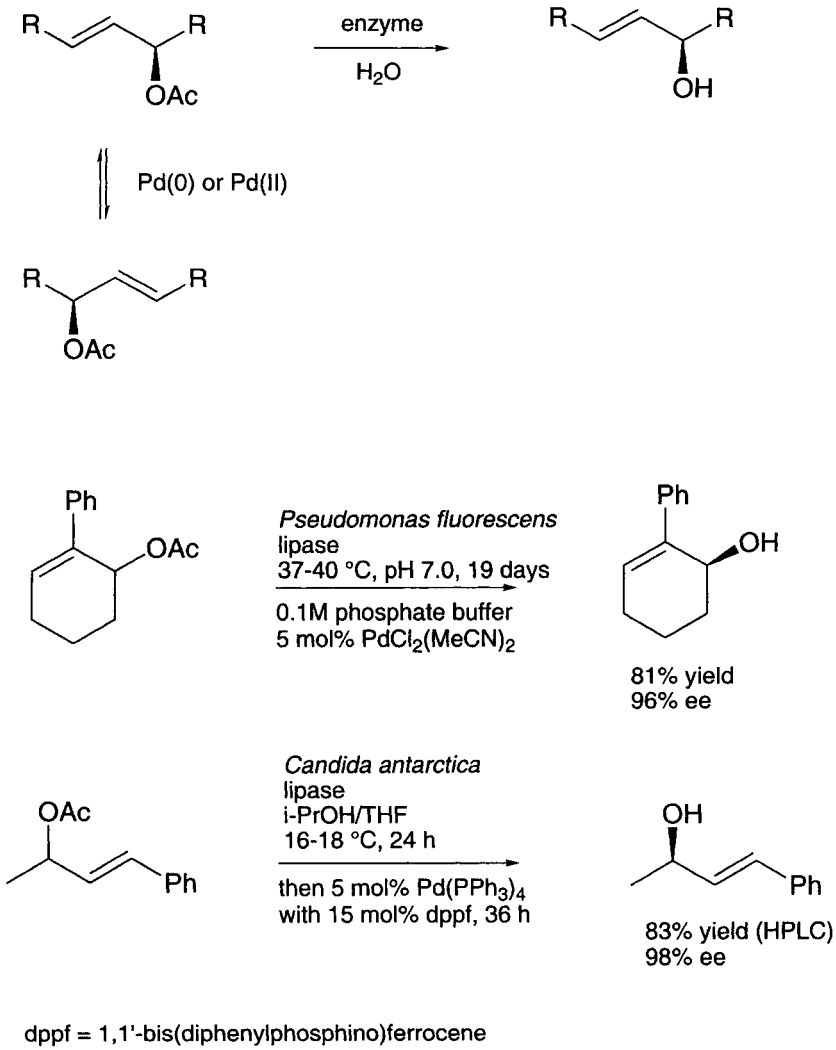
9.2.3
Enzyme and Metal Combinations

Most substrates for enzyme-catalyzed kinetic resolution reactions do not undergo spontaneous racemization under conditions that are suitable for enzyme activity. One solution to this problem has been to design mild transition metal-catalyzed methods for *in situ* racemization[17]. In order to achieve this goal, the racemisation method must be able to function without an adverse effect on the enzyme. Additionally, the enzyme must not inhibit the racemization method.

The first example of the use of enzyme and metal combinations to provide a dynamic resolution procedure was reported by Allen and Williams in 1996[18]. In this case, a palladium (II) catalyst was employed that was able to racemize the allylic acetate substrate, but did not erode the enantioselectivity of the product allylic alcohol (Fig. 9-11). For example, a cyclic acetate was shown to undergo a simple kinetic resolution, affording enantiomerically enriched starting material and product at approximately 50 % conversion. However, performing the reaction in the presence of a palladium (II) catalyst facilitated a dynamic resolution by continuously racemizing the starting material as the reaction progressed.

Similar methodology was applied to an acyclic allylic acetate by the group of Kim, who used Pd(0) catalysts[19]. Acyclic allylic acetates are easier substrates for palladium-catalyzed racemization, and these workers were able to effect a dynamic resolution strategy within a more acceptable time scale (Fig. 9-11). The *in situ* racemization with palladium catalysts is limited in scope, since allylic acetates are required as substrates. In addition, not all allylic acetates are expected to undergo facile racemization[20].

An alternative enzyme/transition metal combination employs transfer hydrogenation catalysts that are capable of racemizing secondary alcohols. The racemization procedure temporarily converts the alcohol into an achiral ketone, which is reduced back to the racemic alcohol. Coupling this racemization procedure to an enzyme-catalyzed acylation reaction affords a dynamic resolution process (Fig. 9-12). Several enzyme/transition metal combinations have been shown to be effective for these reactions, although ruthenium complexes **1**-**3** appear to be especially effective for the *in situ* racemization of the alcohol. The product esters are not prone to racemization under the reaction conditions. Early results employing transfer hydrogenation catalysts to effect the racemization of alcohols required the use of added ketone[21, 22]. However, it was subsequently shown that added ketone was not required when appropriate transition metal complexes were used as catalysts. Furthermore, the use of 4-chlorophenyl acetate as the acyl donor afforded improved results.



dppf = 1,1'-bis(diphenylphosphino)ferrocene

Figure 9-11. Dynamic resolution using transition metal/enzyme combinations.

Bäckvall and co-workers have reported successful results for a wide range of substrates, some of which are identified in Table 9-1. The procedure works well for secondary alcohols containing aryl and alkyl groups[23], diols[24] and α-hydroxy esters[25]. Although catalyst **1** requires no additional base, Kim, Park and co-workers used triethylamine to facilitate racemization using catalyst **2**, Table 9-2[26]. In their case, small quantities of oxygen were added to initiate the racemization procedure. In the case of allylic alcohols, careful choice of racemisation catalyst is required in order to minimize the amount of conversion of the substrate into saturated or

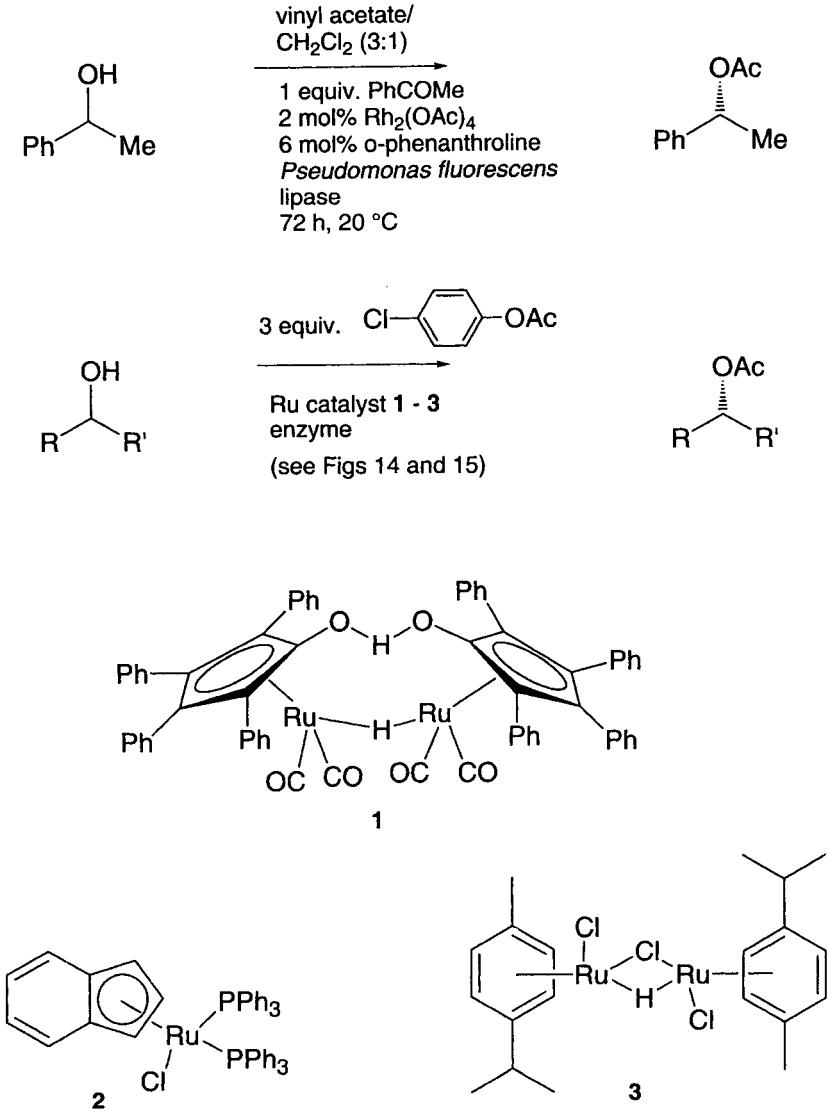
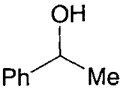
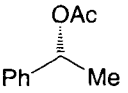
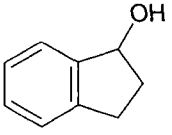
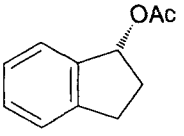
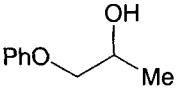
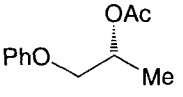
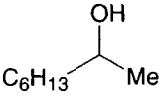
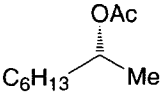
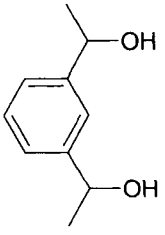
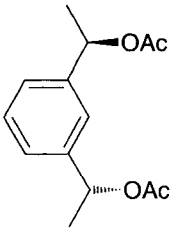
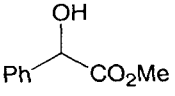
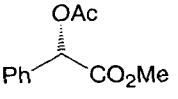
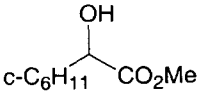
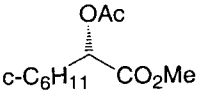


Figure 9-12. Transition metal-catalyzed racemization of alcohols coupled with enantioselective enzyme-catalyzed acetylation.

unsaturated ketones. For allylic alcohols, Kim, Park and co-workers have used catalyst **3**, which minimizes the formation of undesirable side products[27].

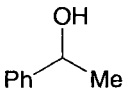
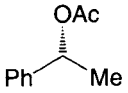
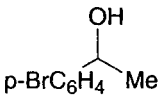
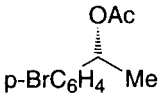
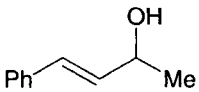
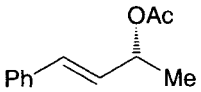
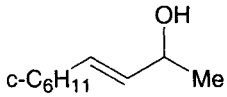
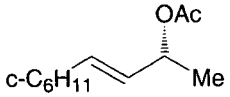
In addition to the use of enzyme and transition metal combinations for the dynamic resolution of alcohols, there has been a brief report of the use of amines as substrates. In 1996, Reetz and Schimossek reported the combination of palladium on carbon with an immobilized lipase (from *Candida antarctica*) in the dynamic

Table 9-1. Examples of dynamic resolution of secondary alcohols with catalyst **1**.

Substrate	Product	Conditions	Yield (%)	ee (%)
		2 mol% Ru cat **1** Novozym 435 3 equiv $pClC_6H_4OAc$ toluene 70 °C, 46 h	80	> 99
		2 mol% Ru cat **1** Novozym 435 3 equiv $pClC_6H_4OAc$ toluene 70 °C, 48 h	77	> 99
		2 mol% Ru cat **1** Novozym 435 3 equiv $pClC_6H_4OAc$ toluene 70 °C, 46 h	88	> 99
		2 mol% Ru cat **1** Novozym 435 3 equiv $pClC_6H_4OAc$ toluene 70 °C, 24 h	80	> 97
		2 mol% Ru cat **1** Novozym 435 3 equiv $pClC_6H_4OAc$ toluene 70 °C, 24 h	77	> 99 (98:2 R,R/meso)
		2 mol% Ru cat **1** PS-C (type II) 2 equiv $pClC_6H_4OAc$ cyclohexane 60 °C, 48 h	80	94
		2 mol% Ru cat **1** PS-C (type II) 2 equiv $pClC_6H_4OAc$ cyclohexane 60 °C, 48 h	80	98

Novozym 435 is *Candida antarctica* lipase B (Novo Nordisk A/S)
PS-C (type II) from Amano is *Pseudomonas cepacia* lipase

Table 9-2. Examples of dynamic resolution of secondary alcohols with catalysts **2** and **3**.

Substrate	Product	Conditions	Yield (%)	ee (%)
		5 mol% Ru cat **2** 5 mol% O_2 PS-C (type II) 3 equiv Et_3N 3 equiv $pClC_6H_4OAc$ CH_2Cl_2, 60 °C, 43 h	85	96
		5 mol% Ru cat **2** 5 mol% O_2 PS-C (type II) 3 equiv Et_3N 3 equiv $pClC_6H_4OAc$ CH_2Cl_2, 60 °C, 43 h	98	99
		4 mol% Ru cat **3** PS-C (type II) 1 equiv Et_3N 1.6 equiv $pClC_6H_4OAc$ CH_2Cl_2, r.t., 48 h	84	> 99
		4 mol% Ru cat **3** PS-C (type II) 1 equiv Et_3N 1.6 equiv $pClC_6H_4OAc$ CH_2Cl_2, r.t., 48 h	90	95

PS-C (type II) from Amano is *Pseudomonas cepacia* lipase

resolution of phenethylamine[28]. The *N*-acylated product was obtained with 99 % *ee* and with 75–77 % yield.

9.3 Carboxylic Acids and their Derivatives

9.3.1 Readily Enolized Carboxylic Acid Derivatives

Carboxylic acid derivatives that have α-substituents can exist as chiral compounds. The resolution of the enantiomers of such compounds is a useful process, leading to the preparation of α-amino acids, α-hydroxy acids and other α-substituted carboxylic acids and their derivatives in enantiomerically enriched form. In addition, the racemization of such compounds can be achieved by a deprotonation/reprotonation sequence, as shown in Fig. 9-13.

The ease with which racemization of the carboxylic acid derivative occurs depends on the nature of the substrate. Carboxylic acids themselves are slow to racemize, since the carboxylic acid is initially deprotonated to form a carboxylate anion.

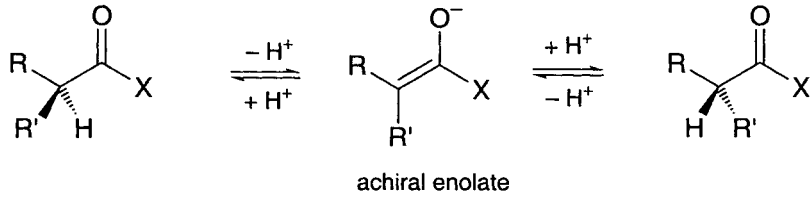


Figure 9-13. Racemization of α-substituted carboxylic acid derivatives by enolization.

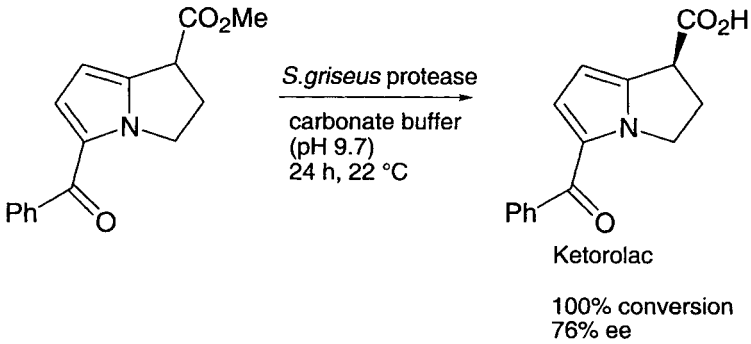


Figure 9-14. Dynamic resolution in the preparation of Ketorolac.

Subsequent deprotonation to afford the carboxylic acid enolate requires the formation of a doubly deprotonated species, which is disfavored relative to the formation of an ester enolate. In fact, activated esters such as phenyl esters[29] or thioesters[30] are especially prone to racemization, since enolization is easier than for simple esters.

Fülling and Sih reported one of the earliest examples to exploit racemization of carboxylic acid derivatives in order to achieve a dynamic kinetic resolution[31]. The anti-inflammatory drug Ketorolac was prepared by hydrolysis of the corresponding ester. Whilst most lipases afforded the undesired enantiomer preferentially, a protease from *Streptomyces griseus* afforded the required (*S*)-enantiomer of product with good selectivity. The substrate was particularly prone to racemization since the intermediate enolate is well stabilized by resonance effects, although a pH 9·7 buffer was required to achieve a useful dynamic resolution reaction. Thus the acid was formed with complete conversion and with 76% enantiomeric excess.

Drueckhammer and co-workers have published details of a successful strategy for dynamic resolution in the hydrolysis of suitable thioesters[30, 32]. Trioctylamine was employed as the racemizing agent, which was effective for the racemization of a series of α-substituted thiopropionates. Specific examples include the hydrolysis of an ethylthioester using *Pseudomonas cepacia* lipase, the transesterification of an α-aryloxy trifluoroethylthioester with butanol and PS-30, as well as hydrolysis of a trifluoroethylthioester using *Subtilisin Carlsberg* (Fig. 9-15).

The ability to achieve dynamic kinetic resolution using thioester substrates has been recognized by other workers, and reports of dynamic resolution strategies

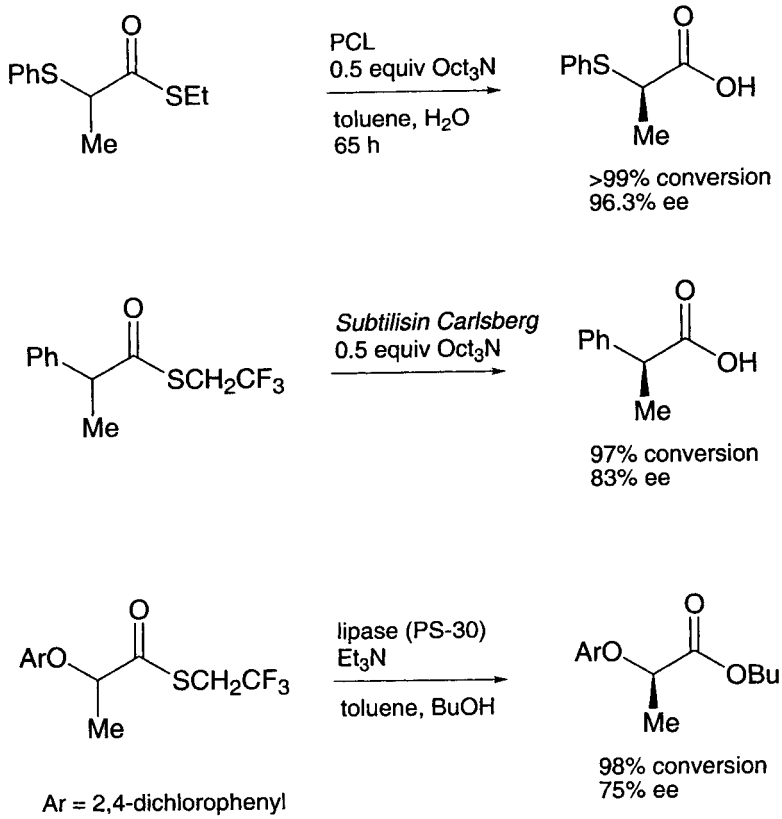


Figure 9-15. Dynamic resolution in the hydrolysis/transesterification of thioesters.

leading to the anti-inflammatory drugs Naproxen[33] and Suprofen[34] have been published. Trioctylamine is again used as the racemizing agent, as shown in Fig. 9-16.

In addition to *in situ* racemization of α-substituted carboxylic acid derivatives by deprotonation/reprotonation, a procedure involving halide exchange has been developed[35, 36]. Whilst the α-halo esters undergo racemization at a reasonable rate, the corresponding carboxylates are almost inert to racemization under the reaction conditions. Using immobilized phosphonium halide and CLEC (cross-linked enzyme crystals), a dynamic resolution procedure has been developed for the hydrolysis of α-bromo and α-chloro esters (Fig. 9-17). The enantiomeric excess in each case was similar to that achieved for simple kinetic resolution reactions using the same enzyme/substrate combinations.

Nitriles can be hydrolyzed by various microorganisms, affording the corresponding carboxylic acids. A method has been reported for the hydrolysis of racemic mandelonitrile (PhCH(OH)CN) into (*R*)-mandelic acid using *Alcaligenes faecalis*

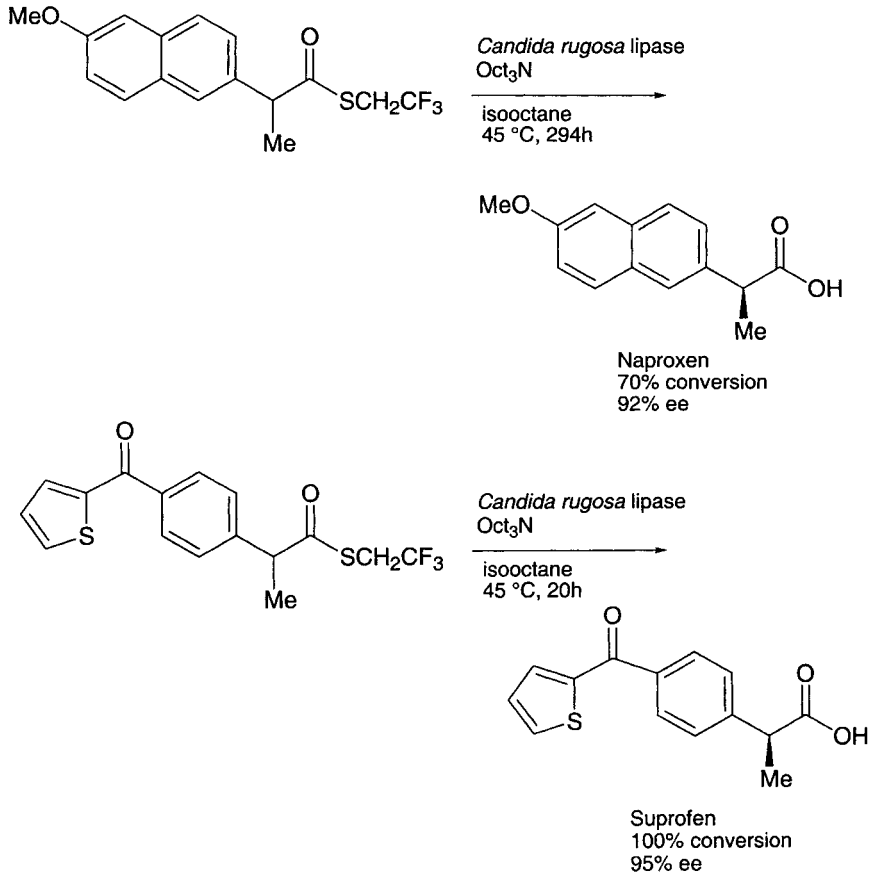


Figure 9-16. Dynamic resolution in the synthesis of Naproxen and Suprofen.

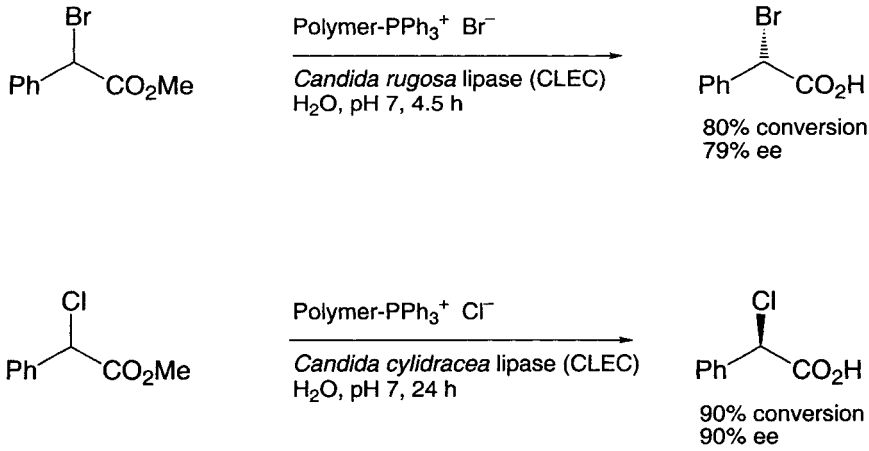


Figure 9-17. Racemization of α-haloesters by halide exchange coupled with enzymatic hydrolysis.

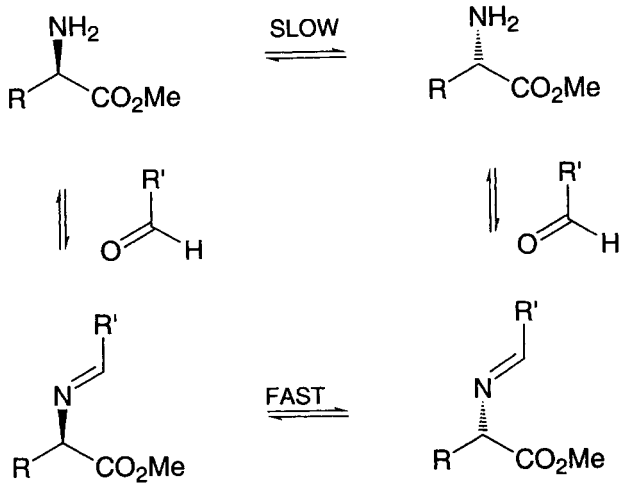


Figure 9-18. Racemization of α-aminoesters catalyzed by imine formation.

ATCC 8750. An isolated yield of 94 % of the enantiomerically pure mandelic acid was obtained, indicating that a dynamic resolution process is occurring.

9.3.2
Amino-Esters and Related Compounds

Typical α-amino esters only undergo racemization slowly, but methods for accelerating this process have been devised[37, 38]. Temporary conversion of the amine to an imine lowers the pK_a of the substrate, such that racemization becomes faster.

A series of α-aminoesters has been hydrolyzed to α-amino acids using alcalase in the presence of pyridoxal 5-phosphate[40]. During the course of these reactions, the amino acids precipitated from the reaction mixture, thereby protecting them from racemisation. The method was used to prepare enantiomerically enriched phenylalanine, leucine, tryptophan and norvaline with high selectivity (Fig. 9-19).

A related ammonolysis of an amino ester has been reported using either pyridoxal or salicylaldehyde as the racemizing agent[41]. The amino ester undergoes racemization more quickly than the amino amide, and an effective dynamic resolution could be achieved at −20 °C.

Pre-formed imino-esters have also been used as substrates for dynamic kinetic resolution reactions[42]. The free amino acid precipitated from the reaction mixture as the reaction proceeded.

α-Azido amides have been subjected to kinetic resolution reactions using whole cells of *E. coli* DH5α/pTrpLAP, affording hydrolysis to the corresponding acids[43]. In the case of 2-azidophenylacetic acid amide, the substrate racemized *in situ*, and the acid product could be obtained with 98 % *ee* at over 50 % conversion.

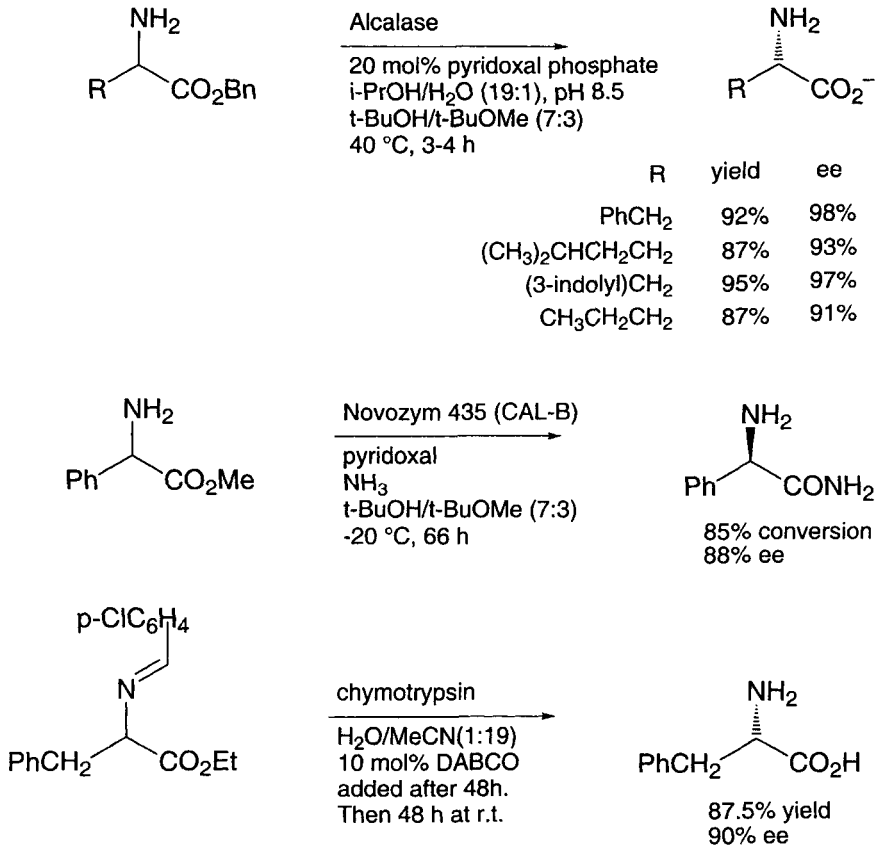


Figure 9-19. Dynamic resolution of amino acids via imine formation.

9.3.3
Reactions of cyclic amino acid derivatives

There are several cyclic amino acids derivatives that are prone to racemization and have been used as substrates for dynamic kinetic resolution reactions.

Oxazolinones were first used as substrates for enzyme-catalyzed hydrolysis over 30 years ago[44]. It was noted that spontaneous hydrolysis could be quite high, depending on the amino acid derivative being used and the pH of the reaction medium[45]. Bevinakatti and co-workers demonstrated that oxazolinones could undergo racemization during a lipase-catalyzed enantioselective ring-opening with *n*-butanol[46, 47]. At 100 % conversion, they were able to obtain (*S*)-butyl *N*-benzoylalaninate with 34 % *ee*.

This concept has been developed by the research groups of Sih and Turner. The oxazolinone derived from phenylalanine was subjected to lipase-catalyzed hydrolysis with ten lipases[48]. Whilst several lipases gave good enantioselectivities, the lipase

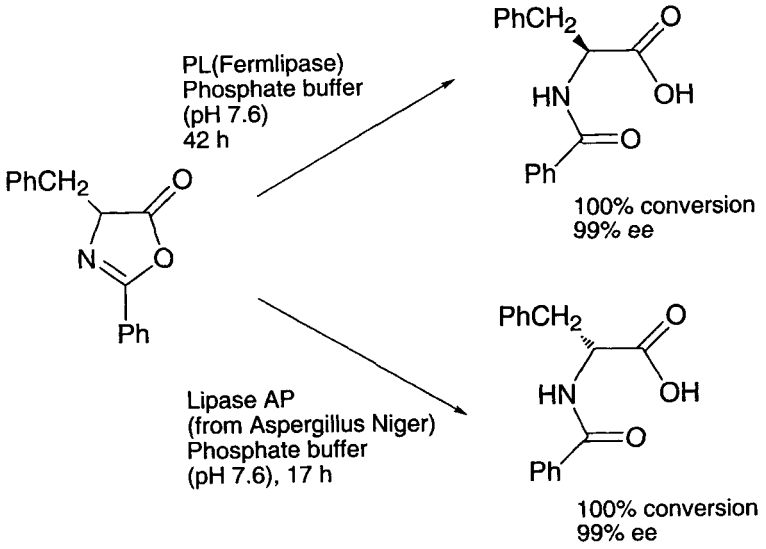


Figure 9-20. Dynamic resolution in the hydrolysis of oxazolinones.

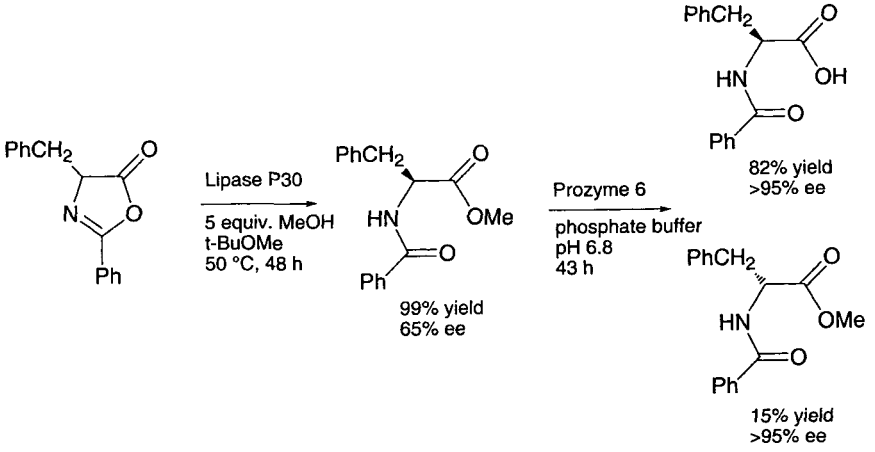
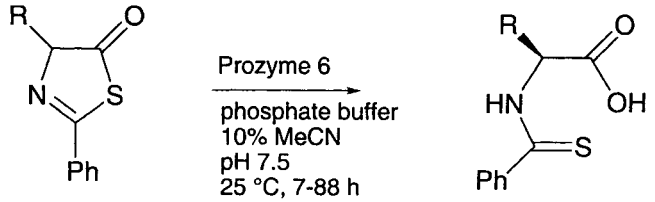


Figure 9-21. Two stage hydrolysis of oxazolinones.

from *Aspergillus niger* (AP) and porcine pancreatic lipase (PL Fermlipase) provided particularly good enantioselectivities, with an opposite sense of asymmetric induction from each other (Fig. 9-20).

An additional strategy employed by Sih and co-workers involved sequential enzyme-catalyzed reactions. *Pseudomonas* lipases were found to tolerate a wide range of substrates although the enantioselectivity was generally only moderate. However, by first performing a methanolysis of the oxazolinone followed by a separate enzyme-catalyzed hydrolysis under kinetic resolution conditions, a highly enantiomerically enriched product could be obtained, as shown in Fig. 9-21[49].



R	yield	ee
Me	67%	90% (No MeCN)
$(CH_3)_2CHCH_2$	78%	97%
$CH_3SCH_2CH_2$	94%	98%
$CH_3CH_2CH_2CH_2$	86%	99%
H_2NCOCH_2	98%	57% (No MeCN)

Figure 9-22. Dynamic resolution in the hydrolysis of thiazolinones.

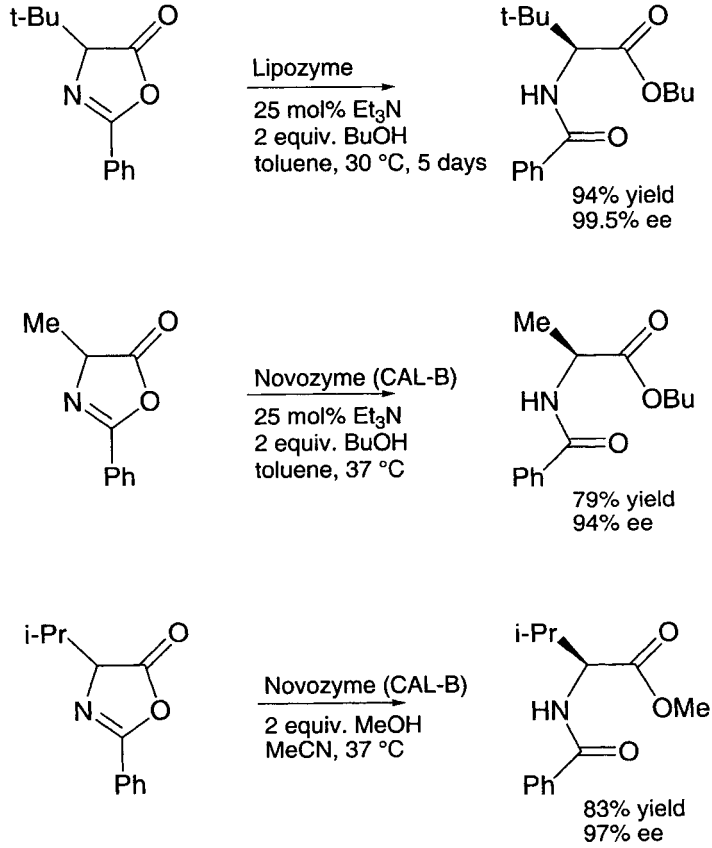


Figure 9-23. Dynamic resolution in the alcoholysis of oxazolinones.

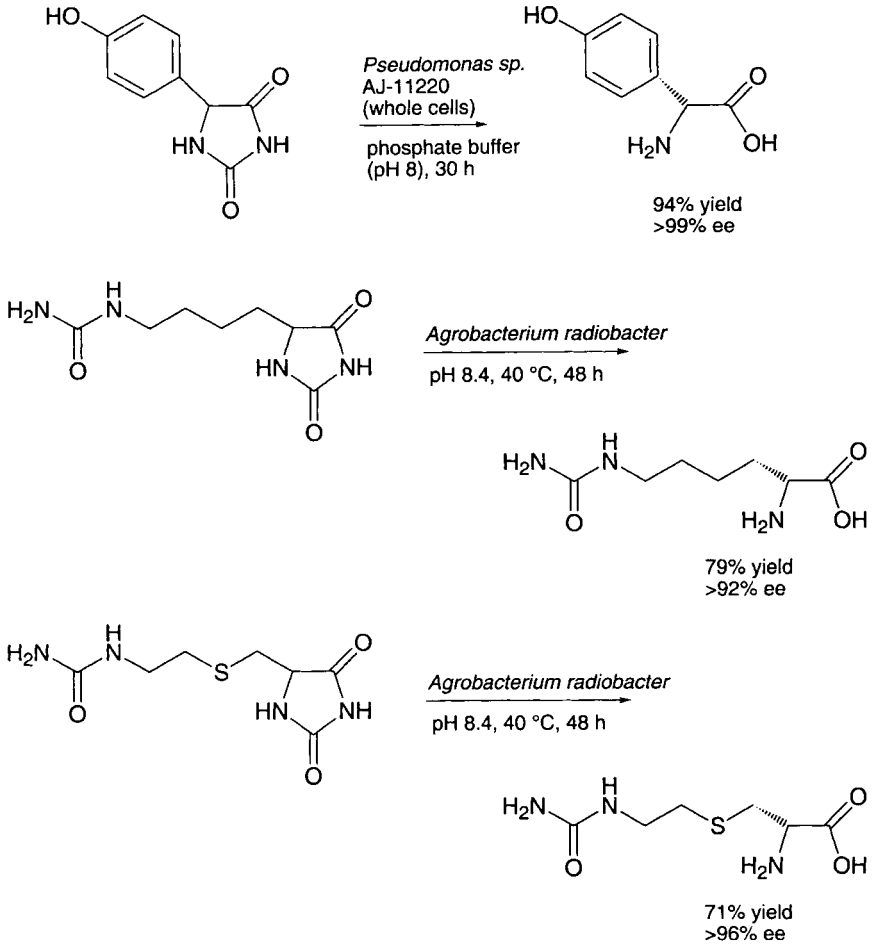


Figure 9-24. Dynamic resolution in the hydrolysis of hydantoins.

Sih and co-workers also reported the dynamic resolution of a range of thiazolinones by enantioselective hydrolysis using proteases[49]. In these cases, the product is the corresponding thioamide. Some of the higher enantiomeric excesses reported are identified in Fig. 9-22.

Turner and co-workers identified conditions appropriate for the dynamic resolution of a 4-*tert*-butyl substituted oxazolinone[50]. The ring-opened butyl ester could be obtained with high yield (94 %) and enantiomeric excess (99·5 %) using Lipozyme – *Mucor miehei* and 0·25 equivalents of triethylamine. Subsequent cleavage of the ester and amide groups afforded a route to enantiomerically pure (*S*)-*tert*-leucine. Whilst Lipozyme provided high selectivities for the sterically demanding *tert*-butyl group, Turner reported that *Candida antarctica* lipase B (Novozyme) was preferred for smaller groups, as shown in Fig. 9-23[51].

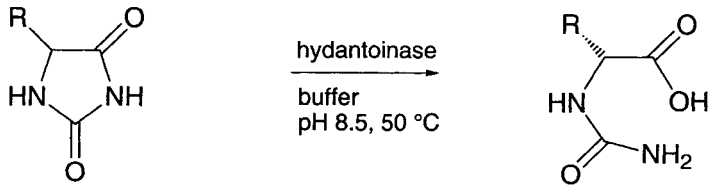


Figure 9-25. Dynamic resolution of racemic hydantoins.

The other major class of cyclic amino acid derivative used in dynamic resolution reactions is the hydantoin group. Like oxazolinones, hydantoins readily undergo racemisation under mild conditions. Systems involving a two step procedure using D-hydantoinase and a carbamoylase were reported to provide a route to D-amino acids[52, 53]. Dynamic resolution of a p-hydroxyphenyl substituted hydantoin was reported in 1987[54]. Using the intact cells of *Pseudomonas* sp. AJ-11220, the amino acid was prepared in over 90% yield, as shown in Fig. 9-24. This hydrolytic procedure leads directly to the amino acid, and the same enantiomer of product, the D-amino acid, was obtained independently of the stereochemistry of the substrate.

A similar strategy has been used in the hydrolysis of hydantoins with pendant ureido groups, using the bacterial culture *Agrobacterium radiobacter*[55].

D-Hydantoinases have also been isolated from thermophilic micro-organisms, and applied to the dynamic resolution of racemic hydantoins, where the isolated products are the *N*-carbamoyl D-amino acids (Fig. 9-25)[56]. Subsequent transformation into D-amino acids could be achieved chemically or enzymatically. Representative examples using commercially available hydantoinases D-HYD-1 and D-HYD-2 are shown in Table 9-3. Variously ring-substituted D-phenylglycine derivatives have also been prepared by hydantoin hydrolysis using D-HYD-1 and D-HYD-2, affording the amino acid with excellent levels of enantioselectivity and good yields[57].

Table 9-3. Dynamic resolution using hydantoinase enzymes.

R	Enzyme	Carbamoylate yield (%)	Amino acid	ee (%)
Me	D-HYD-1	71	94	(D-alanine)
	D-HYD-2	73	34	
$PhCH_2$	D-HYD-1	67	> 99	(D-phenylalanine)
	D-HYD-2	12	> 99	
i-Pr	D-HYD-1	66	> 99	(D-valine)
	D-HYD-2	71	> 99	
$MeSch_2CCH_2$	D-HYD-1	81	> 99	(D-methionine)
	D-HYD-2	75	> 99	
Ph	D-HYD 1	95	96	(D-phenylglycine)
	D-HYD-2	90	> 99	

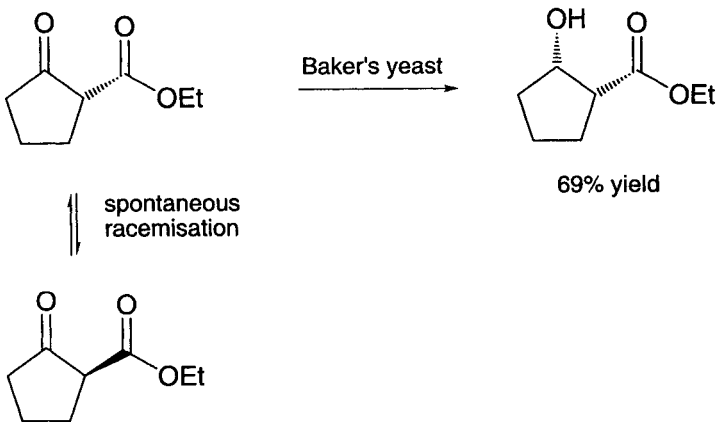


Figure 9-26. Dynamic resolution in the reduction of β-ketoesters.

9.4 Reduction of β-Ketoesters

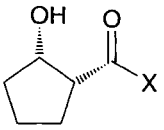
The reduction of ketones into alcohols can be achieved using biocatalytic methods. Amongst the most popular of the available methods is the use of Baker's yeast, BY (*Saccharomyces cerevisiae*). The use of β-ketoesters as substrates leads to the corresponding β-hydroxy esters, often with high enantioselectivity. In the particular case of α-substituted β-ketoesters, the substrates spontaneously racemize, and this provides the basis for many reports of dynamic resolution reactions, some of which are described in the following discussion. In 1976, Deol and co-workers showed that cycloalkyl β-ketoesters could be reduced under dynamic resolution conditions (Fig. 9-26)[58].

In fact, many microorganisms are able to achieve similar reductions on the same and related substrates. Azerad and co-workers have achieved higher selectivities using other microorganisms including *Geotrichum candidum, Mucor racemosus, Kloeckera magna* and *Mucor circinelloides*[59–61]. The opposite diastereomer of product (1*S*,2*S* instead of 1*S*,2*R*) was obtained using *Penicillium chrysogenum* and *Colletotrichum gloeosporoides* as the microorganism. A range of cyclic β-hydroxyesters has been prepared, some of which are identified in Fig. 9-27. The use of a β-ketothioester as a substrate has been reported to afford better stereoselectivity[62].

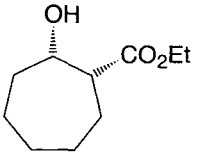
Heterocyclic β-ketoesters have also been used as substrates for reduction, where the products often have use in the synthesis of pharmaceutical agents or natural products. Representative examples of heterocyclic β-hydroxyesters formed using Baker's yeast are given in Fig. 9-28[65–71].

Acyclic β-ketoesters are generally less predictable as substrates than their cyclic counterparts, with the selectivity depending on the nature of the groups attached to the dicarbonyl moiety (Fig. 9-29).

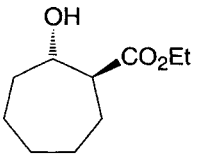
Representative examples of acyclic β-hydroxyesters obtained by dynamic resolu-



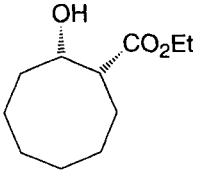
Baker's yeast [62]
X= OMe 93% cis
94% ee
X= SEt 100% cis
>96% ee



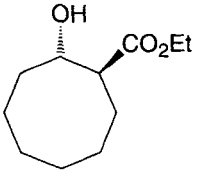
Kloeckera magna [60]
100% cis
99% ee



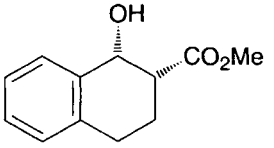
Mucor griseocyanus [60]
88% trans
95% ee



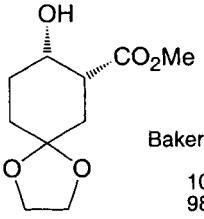
Beauveria bassiana [60]
100% cis
98% ee



Mucor griseocyanus [60]
100% trans
88% ee



Baker's yeast [61]
>98% cis
72% ee



Baker's yeast [63,64]
100% cis
98% ee

Figure 9-27. Cyclic β-hydroxyesters obtained by dynamic resolution.

tion are provided in Fig. 9-30, where Baker's yeast, as well as other microorganisms, have been employed in the reduction process[72–80].

Improved stereocontrol has been obtained using recombinant *E. coli* strains expressing Gre3p or Gcy1p (from Baker's yeast). Since fewer competing enzymes are present in the recombinant *E. coli*, the enantioselectivity and diastereoselectivity are found to be better than using Baker's yeast itself as shown in Fig. 9-31[81].

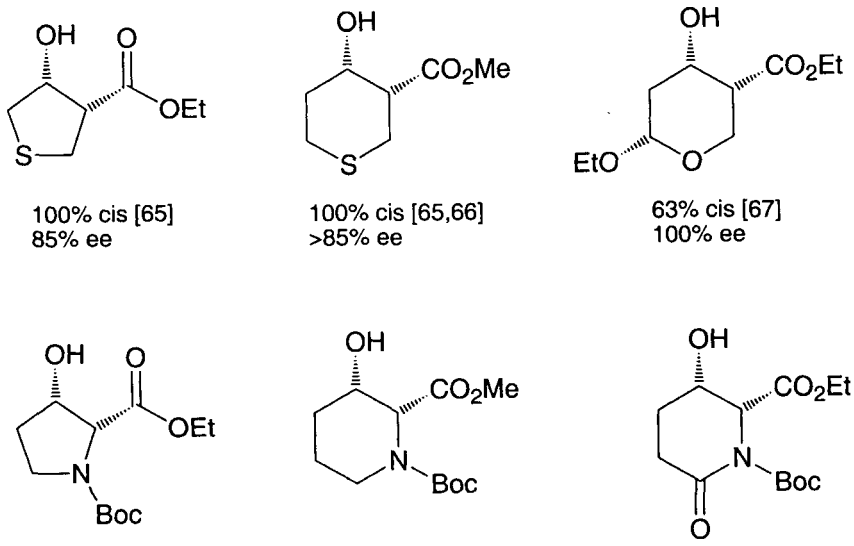


Figure 9-28. Heterocyclic β-hydroxyesters obtained by dynamic resolution.

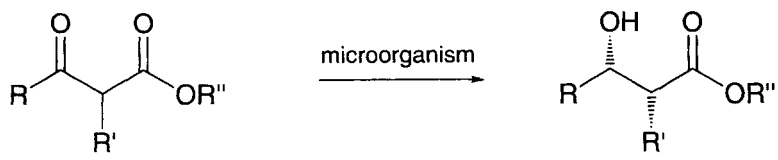


Figure 9-29. Dynamic resolution of acyclic β-ketoesters.

9.5 Conclusion

In summary, dynamic resolution strategies employing biocatalytic methods provide a useful synthetic route to a range of enantiomerically enriched building blocks. Over the last few years there has been a growing interest in finding new methods for the racemization of the starting material. The challenge is to discover racemization methods that are compatible with the biotransformation. Nevertheless, substrates that spontaneously racemize, such as β-ketoesters, still provide the most practicable starting materials for biocatalytic dynamic resolution reactions.

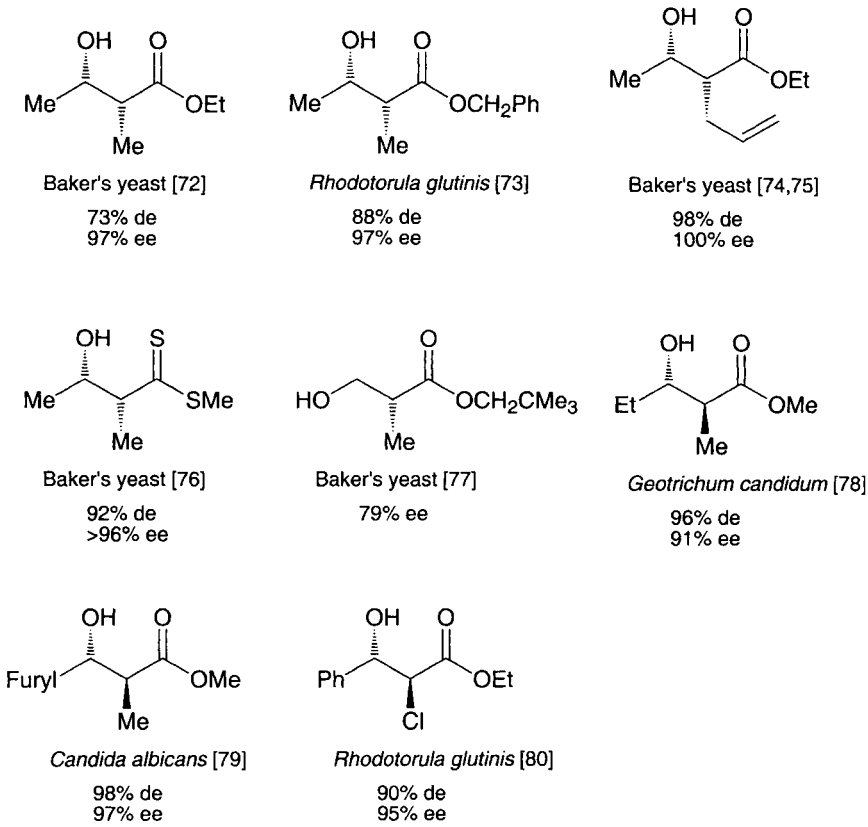


Figure 9-30. Acyclic β-hydroxyesters obtained by dynamic resolution.

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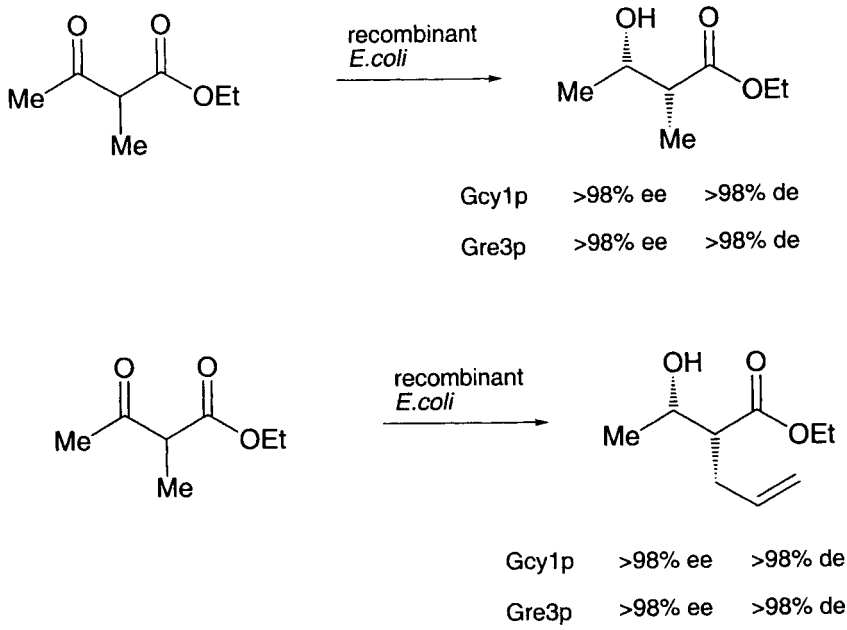

Figure 9-31. Dynamic resolution using recombinant *E. coli*.

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10
Enzymes from Extreme Thermophilic and Hyperthermophilic Archaea and Bacteria

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10.1
Introduction

Environments that are considered by man to be extreme, such as those affected by extremes of temperature, pH and salt content, are colonized by a diverse range of microorganisms. These include an interesting group which are adapted to growth at high temperatures[1]. In the last two decades it has been possible to isolate microorganisms which can grow optimally even above 100 °C[2–5]. The temperature range of growth can be used to define organisms as psychrophiles (–5 to 20 °C), mesophiles (20 to 45 °C), thermophiles (45 to 65 °C), extreme thermophiles (65 to 85 °C) and hyperthermophiles (85 to 110 °C). The majority of the last group, which thrive above the boiling temperature of water, belong to the Archaea. However, some of these microorganisms also belong to the bacterial kingdom. Based on comparisons of partial nucleic acid sequences derived from 16 S and 18 S rRNAs, the two primary kingdoms (prokaryotes and eukaryotes) are reclassified into three, namely Bacteria, Archaea and Eukarya[6]. Archaea are a newly recognized group of organisms with a distinct evolutionary position and unique physiological, biochemical and genetic properties. The thermophilic representatives of the bacteria that optimally live above 65 °C comprise four genera, namely *Thermotoga, Thermosipho, Fervidobacterium* (Thermotogales order) and *Aquifex* (Aquificales order). The temperature optimum for growth of these microorganisms ranges between 65 and 90 °C. On the other hand the thermophilic representatives of the Archaea comprise more than 20 genera, which belong to the following orders: Sulfolobales, Pyrodictiales, Thermoproteales, Thermococcales, Archaeglobales, Thermoplasmales and the methanogens Methanobacteriales and Methanococcales. Table 10-1 describes some of the growth conditions and of the biochemical features of microorganisms capable of surviving at high temperatures[2–12]. The majority of the microorganisms described in Table 10-1 are heterotrophic and anaerobic; only a few are strict autotrophes. Organisms which belong to the Sulfolobales, Aquificales and Thermoplasmales can also live under aerobic conditions. None of these microorganisms, however, can grow optimally at 100 °C. An exception is the archaeon *Pyrobaculum aerophilum*,

Table 10-1. Taxonomy and some biochemical features of bacteria and archaea growing at high temperatures[a].

Order	Genus	Optimal growth temperature (°C)	Heterotrophic (het) autotrophic (aut) facultative autotrophic (f)	Anaerobic (an) aerobic (ae)
BACTERIA				
Thermotogales	*Thermotoga*	70–80	het	an
	Thermosipho	70–75	het	an
	Fervidobacterium	65–70	het	an
Aquifecales	*Aquifex*	90	het	ae/an
ARCHAEA				
Sulfolobales	*Sulfolobus*	65–80	f	ae/an
	Metallosphaera	75	f	ae
	Acidianus	88	aut	ae/an
	Desulfurolobus	80	het	ae/an
Pyrodictales	*Pyrodictium*	100–105	het, aut	an
	Thermodiscus	88	f	an
	Hyperthermus	100	het	an
Thermoproteales	*Thermoproteus*	88	het, f, aut	an
	Thermotilum	88	het	an
	Desulfurococcus	85	het	an
	Staphylothermus	92	het	an
	Pyrobaculum	100	het, f	ae, an
Thermococcales	*Thermococcus*	70–87	het	an
	Pyrococcus	100	het	an
Archaeoglobales	*Archaeoglobus*	83	f	an
Thermoplasmales	*Thermoplasma*	60	het	ae/an

a Methanogenic microorganisms (Methanobacteriales and Methanococcales) with thermophilic representatives are not shown.

which also grows aerobically at 100 °C. Most of these exotic microorganisms have been isolated by Stetter, Zillig and co-workers from various geothermal habitats such as hot springs, sulfataric fields and deep-sea hydrothermal vents. Of great interest are the enzymes that are formed by extreme thermophilic and hyperthermophilic microorganisms. Some of the enzymes that have been recently studied are even active at 140 °C[13]. This short chapter will cover selected enzymes from extreme thermophilic and hyperthermophilic microorganisms that have been described recently. The enzymes from methanogens and thermophilic microorganisms that grow below 70 °C (such as *Bacillus, Clostridium* and *Thermus*) will not be covered. For more detailed information of this rapidly developing field the reader should consult the following reviews][7–10, 12, 14].

10.2
Starch-Processing Enzymes

Starch from cultivated plants represents an ubiquitous and easily accessible source of energy. In plant cells or seeds, starch is usually deposited in the form of large granules in the cytoplasm. Starch is composed exclusively of α-glucose units that are linked by α-1,4- or α-1,6-glycosidic bonds. The two high-molecular-weight components of starch are amylose (15–25 %), a linear polymer consisting of α-1,4-linked glucopyranose residues, and amylopectin (75–85 %), a branched polymer containing, in addition to α-1,4 glycosidic linkages, α-1,6-linked branch points occurring every 17–26 glucose units. α-Amylose chains, which are not soluble in water but form hydrated micelles, are polydisperse, and their molecular weights vary from hundreds to thousands. The molecular weight of amylopectin may be as high as 100 million, and in solution such a polymer has colloidal or micellar forms.

Because of the complex structure of starch, cells require an appropriate combination of hydrolyzing enzymes for its depolymerization to oligosaccharides and smaller sugars such as glucose and maltose. They can be simply classified into two groups: endo-acting enzymes or endo-hydrolases and exo-acting enzymes or exo-hydrolases. Endoacting enzymes, such as α-amylase (α-1,4-glucan-4-glucanohydrolase; E.C. 3.2.1.1), hydrolyze linkages in the interior of the starch polymer in a random fashion, which leads to the formation of linear and branched oligosaccharides.

Exo-acting starch hydrolases include β-amylase, glucoamalase, and α-glucosidase. These enzymes attack the substrate from the nonreducing end, producing small and well-defined oligosaccharides. β-Amylase (E.C. 3.2.1.2), also referred to as α-1,4-D-glucan maltohydrolase or saccharogen amylase, hydrolyzes α-1,4 glucosidic linkages to remove successive maltose units from the non-reducing ends of the starch chains, producing β-maltose by an inversion of the anomeric configuration of the maltose (Fig. 10-1).

α-Glucosidase (E.C. 3.2.1.20), or α-D-glucoside glucohydrolase, attacks the α-1,4 linkages of oligosaccharides that are produced by the action of other amylolytic enzymes. Unlike glucoamylase, α-glucosidase liberates glucose with an α-anomeric configuration.

Enzymes capable of hydrolyzing α-1,6 glycosidic bonds in pullulan are defined as pullulanases. On the basis of substrate specificity and product formation, pullulanases have been classified into two groups: pullulanase type I and pullulanase type II. Pullulanase type I (E.C. 3.2.1.41) specifically hydrolyzes the α-1,6-linkages in pullulan as well as in branched oligosaccharides (debranching enzyme), and its degradation products are maltotriose and linear oligosaccharides, respectively. Pullulanase type I is unable to attack α-1,4-linkages in α-glucans. Pullulanase type II, or amylopullulanase, attacks α-1,6-glycosidic linkages in pullulan and α-1,4-linkages in branched and linear oligosaccharides, converting the latter to small sugars (Fig. 10–1B).

In contrast to the previously described pullulanases, pullulan hydrolases types I and II are unable to hydrolyze α-1,6-glycosidic linkages in pullulan or in branched

substrates. They can attack α-1,4-glycosidic linkages in pullulan, leading to the formation of panose or isopanose. Pullulan hydrolase type I or neopullulanase (E.C. 3.2.1.135) hydrolyzes pullulan to panose (α-6-D-glucosylmaltose). Pullulan hydrolase type II or isopullulanase (E.C. 3.2.1.57) hydrolyzes pullulan to isopanose (α-6-maltosylglucose). Recently, pullulan-hydrolase type III was described, which attacks α-1,4-as well as α-1,6-glycosidic linkages in pullulan (Fig. 10-1).

Cyclodextrin glycosyltransferase (CGTase, E.C. 2.4.1.19), or α-1,4-D-glucan α-4-D-(α-1,4-D-glucano)-transferase, is an enzyme that is generally found in Bacteria and was recently discovered in Archaea. This enzyme produces a series of non-reducing cyclic dextrins from starch, amylose, and other polysaccharides. α-, β-, and γ-cyclodextrins are rings formed by 6, 7, and 8 glucose units that are linked by α-1,4-bonds, respectively (Fig. 10-1).

10.2.1
Thermoactive Amylolytic Enzymes

10.2.1.1
Heat-Stable Amylases and Glucoamylases.

Extremely thermostable α-amylases have been characterized from the hyperthermophilic Archaea *Pyrococcus furiosus*, *Pyrococcus woesei* and *Thermococcus profundus*. The optimal temperatures for the activity of these enzymes are 100 °C, 90 °C and 80 °C, respectively. Thermoactive amylolytic enzymes have been also detected in hyperthermophilic Archaea of the genera *Sulfolobus, Thermophilum, Desulfurococcus, and Staphylothermus*[15–19]. Molecular cloning of the corresponding genes and their expression in heterologous hosts circumvent the problem of insufficient expression in the natural host. The gene encoding an extracellular α-amylase from *P. furiosus* has recently been cloned, and the recombinant enzyme has been expressed in *B. subtilis* and *E. coli*. This is the first report of the expression of an archaeal gene derived from an extremophile in a *Bacillus* strain. The high thermostability of the pyrococcal extracellular α-amylase (thermal activity even at 130 °C) in the absence of metal ions, together with its unique product pattern and substrate specificity, makes this enzyme an interesting candidate for industrial application. In addition, an intracellular α-amylase gene from *P. furiosus* has been cloned and sequenced. It was interesting to note that the four highly conserved regions usually identified in α-amylases are not found in this enzyme. α-Amylases with lower thermostability and thermoactivity have been isolated from the Archaea *Thermococcus profundus, Pyrococcus* sp. KOD1 and the bacteria *Thermotoga maritima* and *Dictioglomus thermophilum*. The genes encoding these enzymes were successfully expressed in *E. coli*. Similar to the amylase from *B. licheniformis*, which is commonly used in liquefaction, the enzyme from *T. maritima* requires Ca^{2+} for activity[20–26]. Further investigations have shown that the extreme hyperthermophilic Archaeon *Pyrodictium abyssi* can grow on various polysaccharides and also secretes a heat-stable amylase (unpublished results).

In contrast to α-amylase, the production of glucoamylase seems to be very rare in

extremely thermophilic and hyperthermophilic Bacteria and Archaea. Among the thermophilic anaerobic Bacteria, glucoamylases have been purified and characterized from *Clostridium thermohydrosulfuricum* 39E, *Clostridium thermosaccharolyticum* and *Thermoanaerobacterium thermosaccharolyticum* DSM 571[27–29]. Recently, it has been shown that the thermoacidophilic Archaea *Thermoplasma acidophilum*, *Picrophilus torridus* and *Picrophilus oshimae* produce heat- and acid-stable glucoamylases. The purified Archaeal glucoamylases are optimally active at pH 2 and 90 °C. Catalytic activity is still detectable at pH 0.5 and 100 °C. This represents the first report on the production of glucoamylases in thermophilic Archaea (unpublished results).

10.2.1.2
α-Glucosidases.

α-Glucosidases are present in thermophilic Archaea and Bacteria. An intracellular α-glucosidase has been purified from *P. furiosus*. The enzyme exhibits optimal activity at pH 5.0 to 6.0 over a temperature range of 105–115 °C; the half life at 98 °C is 48 h. An extracellular α-glucosidase from the thermophilic Archaeon *Thermococcus* strain AN1 was purified and its molecular characteristics determined[30]. The monomeric enzyme (60 kDa) is optimally active at 98 °C. The purified enzyme has a half-life around 35 min, which is increased to around 215 min in the presence of 1% (w/v) dithiothreitol and 1% (w/v) BSA. The substrate preference of the enzyme is: para-nitrophenyl-α -D-glucoside > nigerose > panose > palatinose > isomaltose > maltose and turanose. No activity was found with starch, pullulan, amylose, maltotriose, maltotetraose, isomaltotriose, cellobiose and β-gentiobiose. The enzyme is also active at 130 °C. The gene encoding α-glucosidase from *Thermococcus hydrothermalis* was cloned by complementation of a *Saccharomyces cerevisiae* maltase-deficient mutant strain[31]. The cDNA clone isolated encodes an open reading frame corresponding to a protein of 242 amino acids. The protein shows 42% identity to a *Pyrococcus horikoshii*, unknown ORF, but no similarities were obtained with other polysaccharidase sequences.

10.2.1.3
Thermoactive Pullulanases and CGTases.

Thermostable and thermoactive pullulanases from extremophilic microorganisms have been detected in *Thermococcus celer*, *Desulfurococcus mucosus*, *Staphylothermus marinus* and *Thermococcus aggregans*. Temperature optima between 90 °C and 105 °C, as well as remarkable thermostability even in the absence of substrate and calcium ions, have been observed. Most thermoactive pullulanases identified to date belong to the type II group, which attack α-1,4- and α-1,6-glycosidic linkages. They have been purified from *P. furiosus*, *T. litoralis*, *T. hydrothermalis* and *Pyrococcus* strain ES4[32–37]. Pullulanase type II from *P. furiosus* and *P. woesei* have been expressed in *E. coli*. The unfolding and refolding of the pullulanase from *P. woesei* has been investigated using guanidinium chloride as denaturant. The monomeric enzyme (90 kDa) was

found to be very resistant to chemical denaturation and the transition midpoint for guanidinium chloride-induced unfolding was determined to be 4.86 ± 0.29 M for intrinsic fluorescence and 4.90 ± 0.31 M for far-UV CD changes. The unfolding process was reversible. Reactivation of the completely denatured enzyme (in 7.8 M guanidinium chloride) was obtained upon removal of the denaturant by stepwise dilution; 100% reactivation was observed when refolding was carried out *via* a guanidinium chloride concentration of 4 M in the first dilution step. Particular attention has been paid to the role of Ca^{2+}, which activates and stabilizes this archaeal pullulanase against thermal inactivation. The enzyme binds two Ca^{2+} ions with a K_d of 0.080 ± 0.010 mM and a Hill coefficient H of 1.00 ± 0.10. This cation significantly enhances the stability of the pullulanase against guanidinium chloride-induced unfolding. The refolding of the pullulanase, on the other hand, was not affected by Ca^{2+} [38]. Very recently, the genes encoding the pullulanases from *T. hydrothermalis*, *Desulfurococcus mucosus* and *T. aggregans* have been isolated and expressed in mesophilic hosts. Since the latter enzyme attacks α-1,4 as well as α-1,6 glycosidic linkages in pullulan, it has been classified as pullulan-hydrolase type III. Pullulan is converted to maltotriose, maltose, panose and glucose [40–42]. The aerobic thermophilic bacterium *Thermus caldophilus* GK-24 produces a thermostable pullulanase of type I when grown on starch. This enzyme debranches amylopectin by attacking specifically α-1,6-glycosidic linkages. The pullulanase is optimally active at 75 °C and pH 5.5, is thermostable up to 90 °C, and does not require Ca^{2+} for either activity or stability. The first debranching enzyme (pullulanase type I) from an anaerobic thermophile was identified in the bacterium *Fervidobacterium pennivorans* Ven5, which was cloned and expressed in *E. coli*. In contrast to pullulanase type II from *P. woesei* (specific to both α-1,6 and α-1,4 glycosidic linkages) the enzyme from *F. pennivorans* Ven5 attacks exclusively the α-1,6-glycosidic linkages in polysaccharides. This thermostable debranching enzyme leads to the formation of long-chain linear polysaccharides from amylopectin [44–45].

Thermostable cyclodextrin glycosyltransferases (CGTases) are produced by *Thermoanaerobacter* species, *Thermoanaerobacterium thermosulfurigenes* and *Anaerobranca gottschalkii* [46–48]. Recently, a CGTase, with optimal temperature at 100 °C, waspurified from a newly isolated Archaeon, *Thermococcus* sp. This is the first report of the presence of a thermostable CGTase in a hyperthermophilic Archaeon [49]. The enzyme from this strain has been cloned and sequenced. The gene of 2217 nucleotides encodes a protein with an MW of 83 kDa. The ability of extreme thermophiles and hyperthermophiles to produce heat-stable glycosyl hydrolases is summarised in Table 10-2.

The finding of extremely thermophilic Bacteria and Archaea capable of producing novel thermostable starch-hydrolyzing enzymes is a valuable contribution to the starch-processing industry. By using robust starch-modifying enzymes from thermophiles, innovative and environmentally friendly processes can be developed, aiming at the formation of products of high added value for the food industry. New and enhanced functionality can be obtained by changing the structural properties of starch. In order to prevent retrogradation, starch-modifying enzymes can be used at higher temperatures. The use of the extremely thermostable amylolytic enzymes can

Table 10-2. Starch hydrolyzing enzymes from extreme thermophilic and hyperthermophilic Archaea and Bacteria.

Enzyme	Organism[a]	Enzyme properties			Remarks
		Optimal temperature	Optimal pH	Mw (kDa)	
α-Amylase	*Desulfurococcus mucosus*[85]	100	5.0	–	Purified/cloned
	Pyrococcus furiosus[100]	100	6.5–7.5	129	Purified/cloned/intracellular
		100	7.0	68	Purified/cloned/extracellular
	Pyrococcus sp. KOD1	90	6.5	49.5	Purified/cloned/extracellular
	Pyrococcus woesi[100]	100	5.5	68	Purified/Extracellular
	Pyrodictium abyssi[98]	100	5.0	–	Crude extract[b]
	Staphylothermus marinus[90]	100	5.5	–	Crude extract
	Sulfolobus solfataricus[88]	–	–	240	Extracellular
	Thermococcus celer[85]	90	5.5	–	Crude extract
	Thermococcus profundus DT5432[80]	80	5.5	42	Purified/cloned/"Amy S"
	Thermococcus profundus[80]	80	4.0–5.0	42	Purified/"Amy L"
	Thermococcus aggregans[85]	95	6.5	–	Cloned
	Dyctyoglomus thermophilum Rt46B.1[73]	90	5.5	75	Purified/cloned/cytoplasmic fraction
	Thermotoga maritima MSB8[90]	85–90	7.0	61	Purified/cloned/lipoprotein
Pullulanase type I	*Fervidobacterium pennavorans* Ven5[75]	80	6	190 (93)	Purified/cloned
	Thermotoga maritima MSB8[90]	90	6.0	93 (subunit)	Cloned/type I[b]
	Thermus caldophilus GK24[75]	75	5.5	65	Purified/cell associated
Pullulanase type II	*Desulfurococcus mucosus*[88]	85	5.5	74	Purified/cloned
	Pyrococcus woesei[100]	100	6.0	90	Purified/cloned/cell associated
	Pyrodictium abyssi[98]	100	9.0	–	Crude extract

Table 10-2. (cont.).

Enzyme	Organism[a]	Enzyme properties			Remarks
		Optimal temperature	Optimal pH	Mw (kDa)	
	Thermococcus celer[85]	90	5.5	–	Crude extract
	Thermococcus litoralis[90]	98	5.5	119	Purified/extracell./glycoprotein
	Thermococcus hydrothermalis[80]	95	5.5	128	Purified/extracell./glycoprotein
Pullulan-hydrolase typ III	*Thermococcus aggregans*[85]	100	6.5	83	Purified/cloned
Glucoamylase	*Thermoplasma acidophilum*[60]	90	6.5	141	Purified
	Picrophilus oshimae[60]	90	2.0	140	Purified
	Picrophilus torridus[60]	90	2.0	133	Purified
CGTase	*Thermococcus* sp.[75]	100	7.0	83	Purified
	Thermoanaerobacterium thermosulfurigenes[60]	80	4.0–4.5	68	Purified/cloned/crystallized
	Anaerobranca gottaschallkii[55]	70	8.0	66	Purified
α-Glucosidase	*Thermococcus strain* AN1[80]	130	–	63	Purified/extracell./glycoprotein
	Thermococcus hydrothermalis[80]	–	–	–	Cloned

a Values in brackets give the optimal growth temperature for each organism in °C
b Unpublished results; – not determined

lead to valuable products, which include innovative starch-based materials with gelatin-like characteristics and defined linear dextrins that can be used as fat substitutes, texturizers, aroma stabilizers and prebiotics. CGTases are used for the production of cyclodextrins that can be used as a gelling, thickening or stabilizing agent in jelly desserts, dressing, confectionery, dairy and meat products. Because of the ability of cyclodextrins to form inclusion complexes with a variety of organic molecules, they improve the solubility of hydrophobic compounds in aqueous solution. This is of interest for the pharmaceutical and cosmetic industries[50, 51]. Cyclodextrin production is a multistage process in which starch is first liquefied by a heat-stable amylase, and in the second step a less-thermostable CGTase from *Bacillus* sp. is used. The application of heat-stable CGTase in jet cooking, where temperatures up to 105 °C are achieved, will allow liquefaction and cyclization to take place in one step.

10.3 Cellulose-Hydrolyzing Enzymes

Cellulose commonly accounts for up to 40% of the plant biomass. It consists of glucose units linked by β-1,4-glycosidic bonds with a polymerization grade of up to 15000 glucose units in a linear mode. Although cellulose has a high affinity to water, it is completely insoluble. Natural cellulose compounds are structurally heterogeneous and have both amorphous and highly ordered crystalline regions. The degree of crystallinity depends on the source of the cellulose, and the more highly crystalline regions are more resistant to enzymatic hydrolysis. Cellulose can be hydrolyzed into glucose by the synergistic action of at least three different enzymes: endoglucanase, exoglucanase (cellobiohydrolase) and β-glucosidase. Synonyms for cellulases (E.C. 3.2.1.4) are β-1,4-D-glucan glucano-hydrolases, endo-β-1,4-glucanases or carboxymethyl cellulases. This enzyme is an endoglucanase which hydrolyzes cellulose in a random manner as endo-hydrolase producing various oligosaccharides, cellobiose and glucose. The enzyme catalyzes the hydrolysis of β-1,4-D-glycosidic linkages in cellulose but can also hydrolyze 1,4-linkages in β-D-glucans containing 1,3-linkages.

Exoglucanases, β-1,4-cellobiosidases, exocellobiohydrolases or β-1,4-cellobiohydrolases (E.C. 3.2.1.91) hydrolyze β-1,4-D-glycosidic linkages in cellulose and cellotetraose, releasing cellobiose from the non-reducing end of the chain.

β-Glucosidases (E.C. 3.2.1.21), gentobiases, cellobiases or amygdalases catalyze the hydrolysis of terminal, non-reducing β-D-glucose residues releasing β-D-glucose. These enzymes have a wide specificity for β-D-glucosides. They are able to hydrolyze β-D-galactosides, β-L-arabinosides, β-D-xylosides, and β-D-fucosides.

10.3.1 Thermostable Cellulases

Thermostable cellulases active towards crystalline cellulose are of great biotechnological interest. Several cellulose-degrading enzymes from various thermophilic

organisms have been cloned, purified, and characterized. A thermostable cellulase from *Thermotoga maritima* MSB8 has been characterized[52]. The enzyme is rather small, with a molecular weight (MW) of 27 kDa, and is optimally active at 95 °C and between pH 6.0 and 7.0. Two thermostable endocellulases, CelA and CelB, were purified from *Thermotoga neapolitana*. CelA (MW of 29 kDa) is optimally active at pH 6 at 95 °C, while CelB (MW of 30 kDa) has a broader optimal pH range (pH 6 to 6.6) at 106 °C. The genes encoding these two endocellulases have been identified[53]. Cellulase and hemicellulase genes have been found clustered together on the genome of the thermophilic anaerobic bacterium *Caldocellum saccharolyticum*, which grows on cellulose and hemicellulose as sole carbon sources. The gene for one of the cellulases (*celA*) was isolated and was found to consist of 1751 amino acids. This is the largest cellulase gene described to date[54].

A large cellulolytic enzyme (CelA) with the ability to hydrolyze microcrystalline cellulose was isolated from the extremely thermophilic bacterium *Anaerocellum thermophilum*[55]. The enzyme has an apparent molecular weight of 230 kDa, exhibits significant activity towards Avicel and is most active towards soluble substrates such as CM-cellulose (CMC) and β-glucan. Maximal activity was observed at pH 5–6 and 85–95 °C. The thermostable exoacting cellobiohydrolase from *Thermotoga maritima* MSB8 has an MW of 29 kDa and is optimally active at 95 °C at pH 6.0–7.5 with a half-life of 2 h at 95 °C. The enzyme hydrolyzes Avicel, CM-Cellulose and β-glucan forming cellobiose and cellotriose. A thermostable cellobiase is produced by *Thermotoga* sp. FjSS3-B1[56]. The enzyme is highly thermostable and shows maximal activity at 115 °C at pH 6.8–7.8. The thermostability of this enzyme is salt dependent. This cellobiase is active against amorphous cellulose and CM-cellulose.

Recently, a thermostable endoglucanase, which is capable of degrading β-1,4 bonds of β-glucans and cellulose, has been identified in the Archaeon *Pyrococcus furiosus*. The gene encoding this enzyme has been cloned and sequenced in *E. coli* and has significant amino acid sequence similarities with endoglucanases from glucosyl hydrolases family 12. The purified recombinant endoglucanase hydrolyzes β-1,4- but not β-1,3-glycosidic linkages and has the highest specific activity with cellopentaose and cellohexaose as substrates[57]. In contrast to this, several β-glucosidases have been detected in Archaea. In fact, archaeal β-glucosidases have been found in *Sulfolobus solfataricus* MT4, *S. acidocaldarius*, *S. shibatae* and *P. furiosus*[58–60]. The enzyme from the latter microorganism (homotetramer, 56 kDa/subunit) is very stable and shows optimal activity at 102 °C to 105 °C with a half-life of 3.5 days at 100 °C and 13 h at 110 °C[60]. The β-glucosidase from *S. solfataricus* MT4 has been purified and characterized[61]. The enzyme is a homotetramer (56 kDa/subunit) and very resistant to various denaturants with activity up to 85 °C[62]. The gene for this β-glucosidase has been cloned and overexpressed in *E. coli*[63–65] (Table 10-3).

Cellulose-hydrolyzing enzymes are widespread in Fungi and Bacteria. Less thermoactive cellulases have already found various biotechnological applications. The most effective enzyme of commercial interest is the cellulase produced by *Trichoderma* sp.[66]. Cellulolytic enzymes can be used in alcohol production to improve juice yields and effective color extraction of juices. The presence of cellulases in

Table 10-3. Production of thermoactive cellulases (exoglucanase, B-glycosidase), xylanases (endoxylanase) and chitinase by some representatives of extreme thermophilic and hyperthermophilic Archaea and Bacteria.

Enzyme	Organism[a]	Enzyme properties			Remarks
		Optimal temperature	Optimal pH	Mw (kDa)	
Endoglucanase	*Thermotoga maritima MSB8*[80]	95	6.0–7.5	27	Purified/cloned/cellulase I
	Thermotoga neapolitana[80]	95	6.0	29	Purified/cloned/Cell A
		106	6.0–6.5	30	Purified/cloned/Cell B
Exoglucanase	*Thermotoga maritima MSB8*	95	6.0–7.5	29	Purified/cellulase II
	Thermotoga sp. strain FjSS3-B.1[80]	115	6.8–7.8	36	Purified/cell-associated
	Anaerocellum thermophilum Rt46B.1[100]	85	6.5	31	Cloned
	Pyrococcus furiosus[100]	100	6.0	35.9	Cloned
β-Glycosidase	*Pyrococcus furiosus*[100]	102–105	–	230/58	Purified/cloned
	Sulfolobus solfataricus[88]	105	5.3	240/56	Purified/cloned
	Thermotoga maritima MSB8	75	6.2	95(47)	Purified/cloned
	Thermotoga sp. strain FjSS3-B.1[80]	80	7.0	100(75)	Purified/toga-associated
Endoxylanases	*Pyrodictium abyssi*[98]	110	5.5	–	Crude extract
	Dyctyoglomus thermophilum Rt46B.1[73]	85	6.5	31	Purified/cloned
	Thermotoga maritima MSB8[80]	92	6.2	120	Pur./toga associated/XynA
		105	5.4	40	Pur./toga-associated/XynB
	Thermotoga sp. strain FjSS3-B.1[80, 85]	105	5.3	31	Pur./cloned/toga-associated
		85	6.3	40	Pur./cloned
	Thermotoga neapolitana[80]	85	5.5	37	Purified
		95	5.5–6.0	119	Purified/cloned
	Thermotoga thermarum[77]	80	6.0	105/150	Pur./toga-associated/Endoxylanase 1
		90–100	7.0	35	Pur./Endoxylanase 2
Chitinase	*Pyrococcus kodakaraensis*[95]	85	5.0	135	Purified/cloned

a Values in the brackets give the optimal growth temperature for each organism in °C
– not determined

detergents causes color brightening, softening and improvement of particulate soil removal. Cellulase (Denimax® Novo Nordisk) is also used for the "biostoning" of jeans instead of using stones. Other applications of cellulases include the pretreatment of cellulosic biomass and forage crops to improve nutritional quality and digestibility, enzymatic saccharification of agricultural and industrial wastes and production of fine chemicals.

10.4 Xylan-Degrading Enzymes

Xylan is a heterogeneous molecule that constitutes the main polymeric compound of hemicellulose, a fraction of the plant cell wall which is a major reservoir of fixed carbon in nature. The main chain of the heteropolymer is composed of xylose residues linked by β-1,4-glycosidic bonds. Approximately half of the xylose residues have substitution at O-2 or O-3 positions with acetyl, arabinosyl and glucuronosyl groups. The complete degradation of xylan requires the action of several enzymes (for a detailed description see reviews[67] and[68]). The endo-β-1,4-xylanase (E.C. 3.2.1.8), or β-1,4-xylan xylanohydrolase, hydrolyzes β-1,4-xylosydic linkages in xylans, while β-1,4-xylosidase, β-xylosidase, β-1,4-xylan xylohydrolase, xylobiase or exo-β-1,4-xylosidase (E.C. 3.2.1.37) hydrolyzes β-1,4-xylans and xylobiose by removing the successive xylose residues from the non-reducing termini. α-Arabinofuranosidase or arabinosidase (E.C. 3.2.1.55) hydrolyzes the terminal non-reducing α-L-arabinofuranoside residues in α-L-arabinosides. The enzyme also acts on α-L-arabinofuranosides [α-L-arabinans containing either (1,3) or (1,5)-linkages]. Glucuronoarabinoxylan endo-β-1,4-xylanase, feraxan endoxylanase or glucuronoarabinoxylan β-1,4-xylanohydrolase (E.C. 3.2.1.136) attacks β-1,4-xylosyl linkages in some glucuronoarabinoxylans. This enzyme also shows high activity toward feruloylated arabinoxylans from cereal plant cell walls. Acetyl xylan esterase (E.C. 3.1.1.6) removes acetyl groups from xylan.

10.4.1 Thermostable Xylanases

So far, only a few extreme thermophilic microorganisms are able to grow on xylan and secrete thermoactive xylanolytic enzymes (Table 10-3). Members of the order Thermotogales and *Dictyoglomus thermophilum* Rt46B.1 have been described to produce xylanases that are active and stable at high temperatures. The most thermostable endoxylanases that have been described so far are those derived from *Thermotoga* sp. strain FjSS3-B.1, *Thermotoga maritima*, *T. neapolitana* and *T. thermarum*. These enzymes, which are active between 80 and 105 °C, are mainly cell-associated and most probably localized within the toga. Several genes encoding xylanases have already been cloned and sequenced. The gene from *T. maritima*, encoding a thermostable xylanase, has been cloned and expressed in *E. coli*. Comparison between the *T. maritima* recombinant xylanase and the commercially

available enzyme, Pulpenzyme™ indicates that the thermostable xylanase could be of interest for application in the pulp and paper industry. A xylanase has been found in the Archaeon *Thermococcus zilligii* strain AN1, which grows optimally at 75 °C. The enzyme has a molecular weight of 95 kDa and a unique N-terminal sequence[68–75]. The pH optimum for activity is 6.0, and the half-life at 100 °C is 8 min. Another archaeal xylanase with a temperature optimum of 110 °C was found in the hyperthermophilic Archaeon *Pyrodictium abyssi*.

Xylanases from Bacteria have a wide range of potential biotechnological applications. They are already produced on an industrial scale and are used as food additives in poultry, for increasing feed efficiency diets[76, 77] and in wheat flour for improving dough handling and the quality of baked products[78]. In recent years, the major interest in thermostable xylanases is found in enzyme-aided bleaching of paper[79]. More than 2 million tons of chlorine and chlorine derivatives are used annually in the United States for pulp bleaching. The chlorinated lignin derivatives generated by this process constitute a major environmental problem caused by the pulp and paper industry[79]. Recent investigations have demonstrated the feasibility of enzymatic treatment as an alternative to chlorine bleaching for the removal of residual lignin from pulp[80]. Treatment of craft pulp with xylanase leads to a release of xylan and residual lignin without undue loss of other pulp components. Xylanase treatment at elevated temperatures opens up the cell wall structure, thereby facilitating lignin removal in subsequent bleaching stages. Xylanases from moderate thermophilic microorganisms are rapidly denatured at temperatures above 70 °C. Several of the non-chlorine bleaching stages used in commercial operations are performed well above this temperature; consequently, the pulp must be cooled before treatment with the available enzymes and reheated for subsequent processing steps[75].

10.5 Chitin Degradation

Chitin is a linear β-1,4 homopolymer of *N*-acetyl-glucosamine residues and is one of the most abundant natural biopolymers on earth. Particularly in the marine environment, chitin is produced in enormous amounts, and its turnover is due to the action of chitinolytic enzymes. Chitin is the major structural component of most fungi and invertebrates[81, 82], while for soil or marine Bacteria chitin serves as a nutrient. Chitin degradation is known to proceed with the endo-acting chitin hydrolase (chitinase A: E.C. 3.2.1.14) and the chitin oligomer-degrading exo-acting hydrolases (chitinase B) and *N*-acetyl-D-glycosaminidase (trivial name: chitobiase; E.C. 3.2.1.52).

Chitobiase degrades only small *N*-acetyl-D-glucosamine oligomers (up to pentamers), and the released *N*-acetyl-D-glucosamine monomers retain their C1 anomeric configuration.

Chitin and its derivatives exhibit interesting properties that make them a valuable raw material for several applications[83–87]. It has been estimated that the annual world-wide formation rate and steady state amount of chitin is in the order of 10^{10} to

10^{11} tons per year. Therefore, application of thermostable chitin-hydrolyzing enzymes (chitinases) is expected for effective utilization of this abundant biomass. Although a large number of chitin-hydrolyzing enzymes have been isolated and their corresponding genes have been cloned and characterized, only few thermostable chitin-hydrolyzing enzymes are known. These enzymes have been isolated from the thermophilic microorganisms *Bacillus licheniformis* X-7u, *Bacillus* sp. BG-11 and *Streptomyces thermoviolaceus* OPC-520[88, 89].

The extreme thermophilic anaerobic Archeon *Thermococcus chitonophagus* has been reported to hydrolyze chitin[90]. This is the first extremophilic Archaeon which produces chitinase(s) and *N*-acetylglucosaminidase(s); however, sequence and structural information for archaeal chitinases have not yet been reported. Very recently, the gene encoding a chitinase from a hyperthermophilic archaeon *Pyrococcus kodakaraensis* KOD1 was cloned, sequenced and expressed in *E. coli*. The purified recombinant protein is optimally active at 85 °C and pH 5.0. The enzyme produces chitobiose as the major end product (Table 10-3).

10.6 Proteolytic Enzymes

Proteins are the most abundant organic molecules in living cells and constitute more than 50% of their dry weight. The molecular weight of proteins that are made up of one or more polypeptide chains can vary from a few thousands to more than one million daltons. All proteins are constructed from a basic set of 20 amino acids that are covalently linked by peptide bonds. The three-dimensional conformation of proteins may vary. Globular proteins (spherical or globular) are soluble and usually have dynamic function. Fibrous proteins on the other hand occur as sheets or rods, are insoluble and serve as structural elements. The enzymes which hydrolyze the peptide bonds in proteins are defined as proteases. They are also called endopeptidases because they hydrolyze peptide bonds inside the polypeptide chain. Exopeptidases (either carboxypeptidases or aminopeptidases) on the other hand can split off the terminal residues of the polypeptide chain. Proteases (endopeptidases) play an important role in the utilization of proteins by various microbes. They are classified into four groups depending on the nature of their active center.

I. Serine proteases have a serine residue in their active center and are inhibited by DFP (diisopropylphosphofluoride) and PMSF (phenylmethylsulfonylfluoride).
II. Cysteine proteases have a SH groups in their active center and are inhibited by thiol reagents, heavy metal ions, alkylating agents and oxidizing agents.
III. The activity of metal proteases depends on tightly bound divalent cations. They are inactivated by chelating agents.
IV. Aspartic proteases (acid proteases) are rare in Bacteria and contain one or more aspartic acid residues in their active center. Inactivation of the enzyme can be achieved by alkylation of the aspartic acid residues with DAN (diazoacetyl-DL-norleucine methyl ester)[91].

10.6.1
Stable proteases

A variety of heat-stable proteases have been identified in hyperthermophilic Archaea belonging to the genera *Desulfurococcus*, *Sulfolobus*, *Staphylothermus*, *Thermococcus*, *Pyrobaculum* and *Pyrococcus*. It has been found that most proteases from extremophiles belong to the serine type and are stable at high temperatures even in the presence of high concentrations of detergents and denaturing agents (Table 10-4). A heat-stable serine protease was isolated from cell-free supernatants of the hyperthermophilic Archaeon *Desulfurococcus* strain $Tok_{12}S_1$[92]. A cell-associated serine protease was characterized from *Desulfurococcus* strain SY that showed a half-life of 4.3 h at 95 °C[93]. A globular serine protease from *Staphylothermus marinus* was found to be extremely thermostable. This enzyme, which is bound to the stalk of filiform glycoprotein complex, named tetrabrachion, has a residual activity even at 135 °C after 10 min of incubation[94]. The properties of extracellular serine proteases from a number of *Thermococcus* species have been analyzed[95]. The extracellular enzyme from *T. stetteri* has a molecular weight of 68 kDa and is highly stable and resistant to chemical denaturation, as illustrated by a half-life of 2.5 h at 100 °C and retention of 70% of its activity in the presence of 1% SDS[96]. A novel intracellular serine protease (perinilase) from the aerobic hyperthermophilic Archaeon *Aeropyrum pernix* K1 was purified and characterized. At 90 °C, the pernilase has a broad pH profile and an optimum at pH 9.0 for peptide hydrolysis. Several proteases from hyperthermophiles have been cloned and sequenced, but in general their expression in a mesophilic host is difficult. A gene encoding a subtilisin-like serine protease, named aereolysin, has been cloned from *Pyrobaculum aerophilum*, and the protein was modeled based on structures of subtilisin-type proteases[97]. Multiple proteolytic activities have been observed in *P. furiosus*. The cell-envelope associated serine protease of *P. furiosus*, called pyrolysin, was found to be highly stable, with a half-life of 20 min at 105 °C[98]. The pyrolysin gene was cloned and sequenced, and it was shown that this enzyme is a subtilisin-like serine protease[99]. A serine protease from *Aquifex pyrophilus* was cloned and weakly expressed in *E. coli*. The activity of the enzyme was highest at 85 °C and pH 9. The half-life of the protein (6 h at 105 °C) makes it one of the most heat-stable proteases known to date.

Proteases have also been characterized from the thermoacidophilic Archaea *Sulfolobus solfataricus* and *S. acidocaldarius*. In addition to the serine proteases, other types of enzymes have been identified in extremophiles: a thiol protease from *Pyrococcus* sp. KOD1, a propylpeptidase (PEPase) and a new type of protease from *P. furiosus*. An extracellular protease, which is designated aeropyrolysin, was purified from *Aeropyrum pernix* K1 (JCM 9820). The enzyme activity is completely inhibited by EDTA and EGTA, indicating that it is a metalloprotease. The enzyme is highly resistant to denaturing reagents and highly thermostable, showing a half-life of 2.5 h at 120 °C and 1.2 h at 125 °C in the presence of 1 mM $CaCl_2$. These results indicate that this enzyme is one of the most thermostable extracellular metallo-proteases reported to date. Thermostable serine proteases were also detected in a number of extreme thermophilic Bacteria belonging to the genera *Thermotoga* and *Fervido-*

Table 10-4. Properties of thermoactive proteolytic enzymes from extreme thermophilic and hyperthermophilic Archaea and Bacteria.

Enzyme	Organism[a]	Enzyme properties			Remarks
		Optimal temperature	Optimal pH	Mw (kDa)	
Serine protease	*Desulfurococcus mucosus*[85]	95	7.5	52	Purified
	Pyrococcus furiosus[100]	85	6.3	124(29) 105/80	Protease I/purified Pyrolysin/pur./cloned
	Pyrobaculum aerophilum[95]	–	–	–	Cloned
	Thermococcus aggregans[75]	90	7.0	–	Crude extract
	Thermococcus celer[85]	95	7.5	–	Crude extract
	Thermococcus litoralis[90]	95	9.5	–	Crude extract
	Thermococcus stetteri[75]	85	8.5	68	Pur./cloned
	Staphylothermus marinus[90]	–	9.0	140	Stable up to 135 °C
	Sulfolobus solfataricus[88]	–	6.5–8	118(52)	Purified
	Aeropyrum Pernix K1[90]	90	9.0	50	Purified
	Aquifex pyrophilus[90]	85	7.0–9.0	43	Purified
	Fervidobacterium pennavorans[70]	80	10	130	Purified/keratin hydrolysis
	Thermobacteroides proteolyticus[65]	85	9.0–9.5	–	Crude extract
Thiol protease	*Pyrococcus* sp. KOD1[95]	110	7	44	Purified
Acidic protease	*Sulfolobus acidocaldarius*[70]	90	2.0	–	Cloned
Aminopeptidase I	*Solfolobus solfataricus*[88]	–	–	> 450	Crude extract
Aminopeptidase II		–	–	170	Crude extract
Endopeptidase I, II, III		–	–	115, 32, 27	Crude extract
Carboxypeptidase		–	–	160	Crude extract

a Values in the brackets give the optimal growth temperature for each organism in °C
– not determined

bacterium (unpublished results). The enzyme system from *Fervidobacterium pennivorans* is able to hydrolyze feather keratin forming amino acids and peptides. The enzyme is optimally active at 80 °C and pH 10.0[109]. The amount of proteolytic enzymes produced worldwide on a commercial scale exceeds that of the other biotechnological enzymes used. Heat-stable proteases have great potential for various applications including the textile and pharmaceutical industries. Serine alkaline proteases are currently used as additives to household detergents for laundering, where they have to resist denaturation by detergents and alkaline conditions. Proteases showing high keratinolytic and elastolytic activities are used for soaking in the leather industry. Proteases are also used as catalysts for peptide synthesis using their reverse reaction[100–109].

10.7 Intracellular Enzymes

A number of intracellular enzymes from extreme thermophilic and hyperthermophilic microorganisms have been investigated. The majority of the intracellular enzymes known to date show slightly less thermostability than the extracellular enzymes. Some of these enzymes, which have been characterized from Archaea belonging to the order Sulfolobalses and Themococcales, include alcohol dehydrogenase, glucose dehydrogenase, glyceraldehyde-3-phospho-dehydrogenase, NADH dehydrogenase, β-galactosidase, citrate synthase, malic enzyme, fumarase, s-adenosylmethionine synthetase, ATPase, ATP sulfurylase, aspartate aminotransferase, DNA polymerase, RNA polymerase, topoisomerase and polyphosphate kinase (for review see[37]). Other reports are available on extremely thermoactive intracellular enzymes that are even active above 100 °C. Glyceraldehyde-3-phosphate dehydrogenase from the Archaeon *P. woesei* was characterized and the gene was cloned in *E. coli*. This enzyme is strictly phosphate dependent and utilizes either NAD+ or NADP+; the half-life of the enzyme at 100 °C is 44 min[110]. The amino acid composition of glyceraldehyde-3-phosphate dehydrogenase from *P. woesei* was determined and compared with mesophilic and thermophilic Archaea. The primary structure of this enzyme exhibits a high proportion of aromatic amino acid residues and a low proportion of sulfur-containing residues. The glutamate dehydrogenase (GDH) from *P. woesei* and *P. furiosus* was purified and characterized. This enzyme is probably involved in the first step of nitrogen metabolism. GDH from *P. woesei* was purified in a single-affinity chromatography step[111]. It utilizes both NAD^+ and $NADP^+$ as cofactors with a preference for the phosphorylated form. The purified enzyme from both strains is a hexamer with identical subunits of 45 kDa each[112, 113]. Twenty-four N-terminal residues of GDH were determined and used to construct gene-specific DNA probes *via* the polymerase chain reaction[111]. The GDH gene was cloned in *E. coli*. Its nucleotide sequence and amino acid composition were determined. A highly thermoactive glucose isomerase with maximal enzymatic activity at 105 °C was purified from *T. maritima* and characterized[114]. This enzyme could play an important role in the industrial bioconversion of glucose

to fructose. Other remarkable thermoactive enzymes such as hydrogenase[115], aldehyde ferredoxin oxidoreductase[116], and acetyl-Co A synthetase (ADP forming) were detected in *P. furiosus*[117–119].

DNA polymerases (E.C. 2.7.7.7.) are other important intracellular enzymes that play a key role in the replication of cellular information present in all life forms. They catalyze, in the presence of Mg^{2+} ions, the addition of a deoxyribonucleoside 5'-triphosphate onto the growing 3'-OH end of a primer strand, forming complementary base pairs to a second strand. Thermostable DNA polymerases play a major role in a variety of molecular biological applications, e.g. DNA amplification, sequencing or labeling. More than 100 DNA polymerase genes have been cloned and sequenced from various organisms, including thermophilic Bacteria and Archaea. One of the most important advances in molecular biology during the last ten years is the development of a polymerase chain reaction (PCR)[120–122]. The PCR procedure first described utilized the Klenow fragment of *E. coli* DNA polymerase I, which was heat labile and had to be added during each cycle following the denaturation and primary hybridization steps. Introduction of thermostable DNA polymerases in PCR facilitated the automation of the thermal cycling part of the procedure. The DNA polymerase I from the bacterium *Thermus aquaticus*, called *Taq* polymerase, was the first thermostable DNA polymerase characterized[123, 124] and applied in PCR.

A thermostable DNA polymerase from *Thermotoga maritima*[125] was reported to have a 3'-5'-exonuclease activity[126]. Archaeal proofreading polymerases such as *Pwo* pol[127] from *Pyrococcus woesei*[128], *Pfu* pol[129]from *Pyrococcus furiosus*[130], Deep VentTM pol[131] from *Pyrococcus* strain GB-D[132] or Vent- pol[133, 134] from *Thermococcus litoralis*[135] have an error rate that is up to ten times lower than that of *Taq* polymerase. The 9°N-7 DNA polymerase from *Thermococcus* sp. strain 9°N-7 has a fivefold higher 3'-5'-exonuclease activity than *T. litoralis* DNA polymerase[136]. However, *Taq* polymerase was not replaced by these DNA polymerases because of their low extension rates, among other factors. DNA polymerases with higher fidelity are not necessarily suitable for amplification of long DNA fragments because of their potentially strong exonuclease activity[137]. The recombinant KOD1 DNA polymerase from *Pyrococcus* sp. strain KOD1 has been reported to show low error rates (similar values to those of *Pfu*), high processivity (persistence of sequential nucleotide polymerization) and high extension rates, resulting in an accurate amplification of target DNA sequences up to 6 kb[138]. In order to optimize the delicate competition of polymerase and exonuclease activity, the exo-motif 1 of the 9°N-7 DNA polymerase was mutated in an attempt to reduce the level of exonuclease activity without totally eliminating it[135, 139, 140]. Similarly, the PCR performance was optimized by site-directed mutagenesis of the DNA binding motif of the DNA polymerase from *Thermococcus aggregans* and *Sulfolobus solfataricus*[141].

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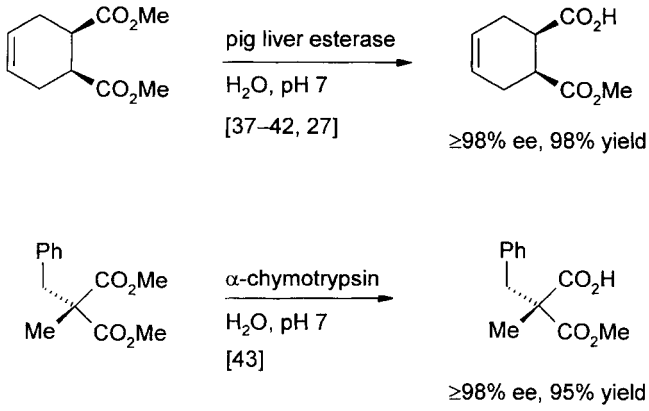
11
Hydrolysis and Formation of C-O Bonds

11.1
Hydrolysis and Formation of Carboxylid Acid Esters

Hans Joachim Gais and Fritz Theil

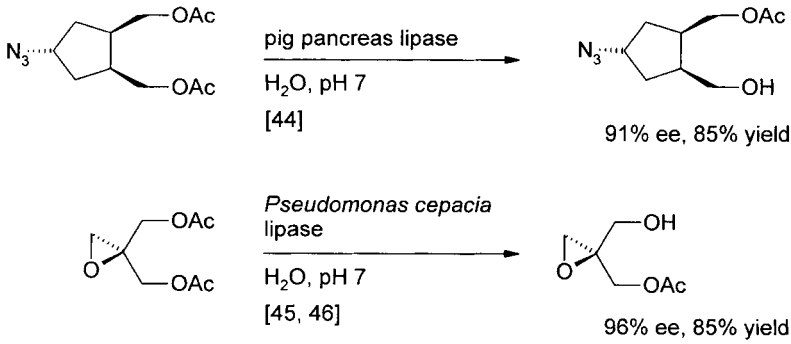
Catalysis of the hydrolysis and formation of the C-O bond of an ester, lactone or carbonate by hydrolases are amongst the most useful enzyme-catalyzed reactions in organic synthesis (*in vitro*)[1-36]. Today hydrolases are established tools for organic synthesis on a laboratory scale as well as on a industrial scale[35]. The reason for this lies in the nature of hydrolases. Hydrolases are chiral catalysts, which are easy to apply[35e], function without a coenzyme, are commercially available in quite a number and variety[35d], and frequently feature low substrate specificity, high enantiotopic selectivity, and enantiomer selectivity. In addition, directed evolution, chemical modification and most importantly site-directed mutagenesis allow for the attainment of enzymes with improved activity, selectivity and stability, in particular toward organic solvents[35f]. This adds considerably to the versatility of hydrolases. The most important application of hydrolases lies in the field of asymmetric synthesis, which is therefore solely dealed with in this chapter. For the application of hydrolases in chemo- and regioselective transformations and in particular in protecting group chemistry, see Chapter 18. Among the hydrolases, the most widely used are, in first place, the lipases (E.C. 3.1.1.3), as for example pig pancreas lipase, *Pseudomonas* sp. lipases and *Candida antarctica* lipase (see Sect. 11.1.1.1.5, Tables 11.1-10 to 11.1-25), in second place the carboxylester hydrolases (E.C. 3.1.1.1), as for example pig liver esterase (see Sect. 11.1.1.1.1., Tables 11.1-1 to 11.1-6 and Sect. 11.1.1.2.3, Table 11.1-27), and in third place the proteases (E.C. 3.4.*m.n*), as for example subtilisin (see Sect. 11.1.1.1.4, Table 11.1-8 and Sect. 11.1.1.2.2, Table 11.1-26) and α-chymotrypsin (see Sect. 11.1.1.1.2, Table 11.1-7). Today, lipases are the most versatile hydrolases, primarily because of their ability to be highly active not only in water but also in water in the presence of an organic cosolvent and, most importantly, even in organic solvents of low water content. Another reason for the versatility of lipases is their accessibility in quite large numbers (see Sect. C). Some confusion has developed in the literature concerning the origin and names of some



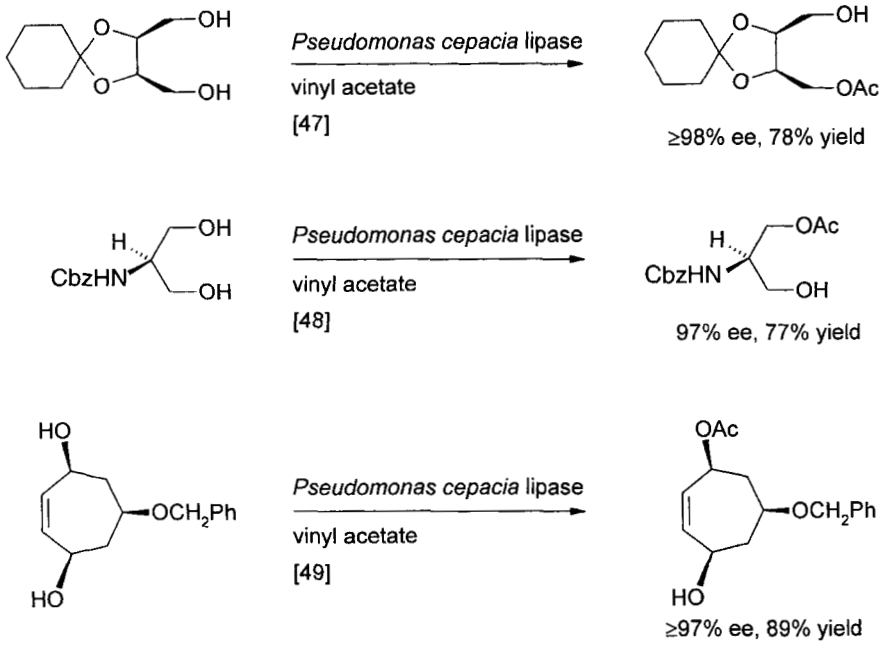
Scheme 11.1-1. Enantiotopos-differentiating hydrolysis of dicarboxylic diesters.

microbial lipases; this topic is dealt with in Sect. 11.1.1.1.5. Appropriate substrates for hydrolases are principally those compounds which bear enantiotopic ester groups with the prochirality contained either in the dicarboxylic acid (Schemes 11.1-1 and 11.1-8) or in the diol part (Schemes 11.1-2 and 11.1-12) of the molecule, or those which carry enantiotopic hydroxyl groups (Schemes 11.1-3 and 11.1-12). A second and no less important class of substrates is the racemates, as for example esters of racemic carboxylic acids (Scheme 11.1-4) or esters of racemic alcohols (Schemes 11.1-5 and 11.1-7), racemic alcohols (Scheme 11.1-6), and racemic hydroxy carboxylic acid esters (Scheme 11.1-8).

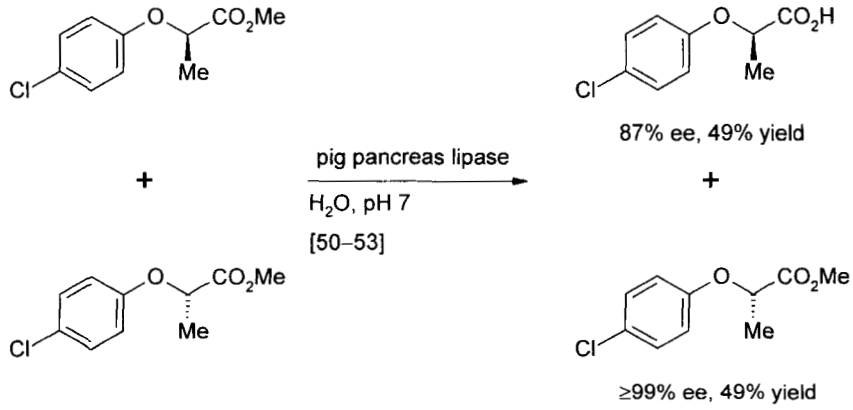
The hydrolase-catalyzed reactions utilized most for the selective transformation of such substrates are hydrolysis (Schemes 11.1-1, 11.1-2, 11.1-4, 11.1-5 and 11.1-11), acylation (transesterification) (Schemes 11.1-3, 11.1-6 and 11.1-11) and alcoholysis (transesterification) (Schemes 11.1-7, 11.1-8 and 11.1-15). Hydrolase-catalyzed esterification of an alcohol with a carboxylic acid, although highly useful in some cases[62], has been utilized to a lesser extent. Catalysis of formation and cleavage of the C-O bond of an ester or lactone by pig liver esterase, most lipases, α-chymotrypsin and subtilisin, which are all serine hydrolases, involves the following steps (Scheme 11.1-9). Formation of an enzyme-substrate complex, attack of the hydroxyl group of



Scheme 11.1-2. Enantiotopos-differentiating hydrolysis of diol diacetates.

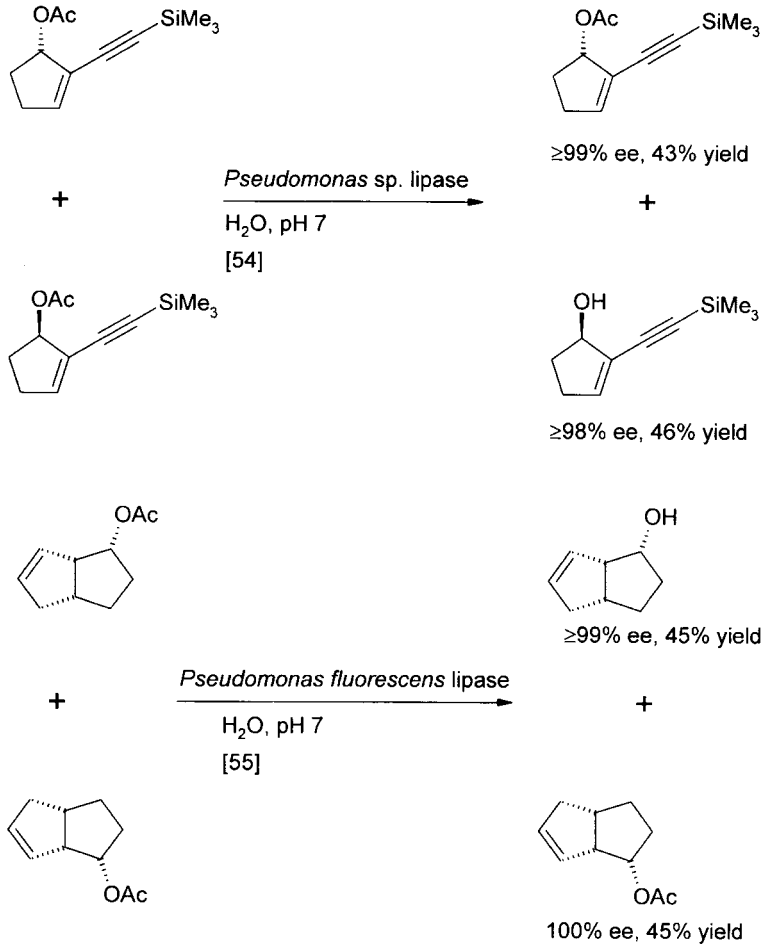


Scheme 11.1-3. Enantiotopos-differentiating transesterification of diols.



Scheme 11.1-4. Enantiotopos-differentiating hydrolysis of carboxylic acid esters.

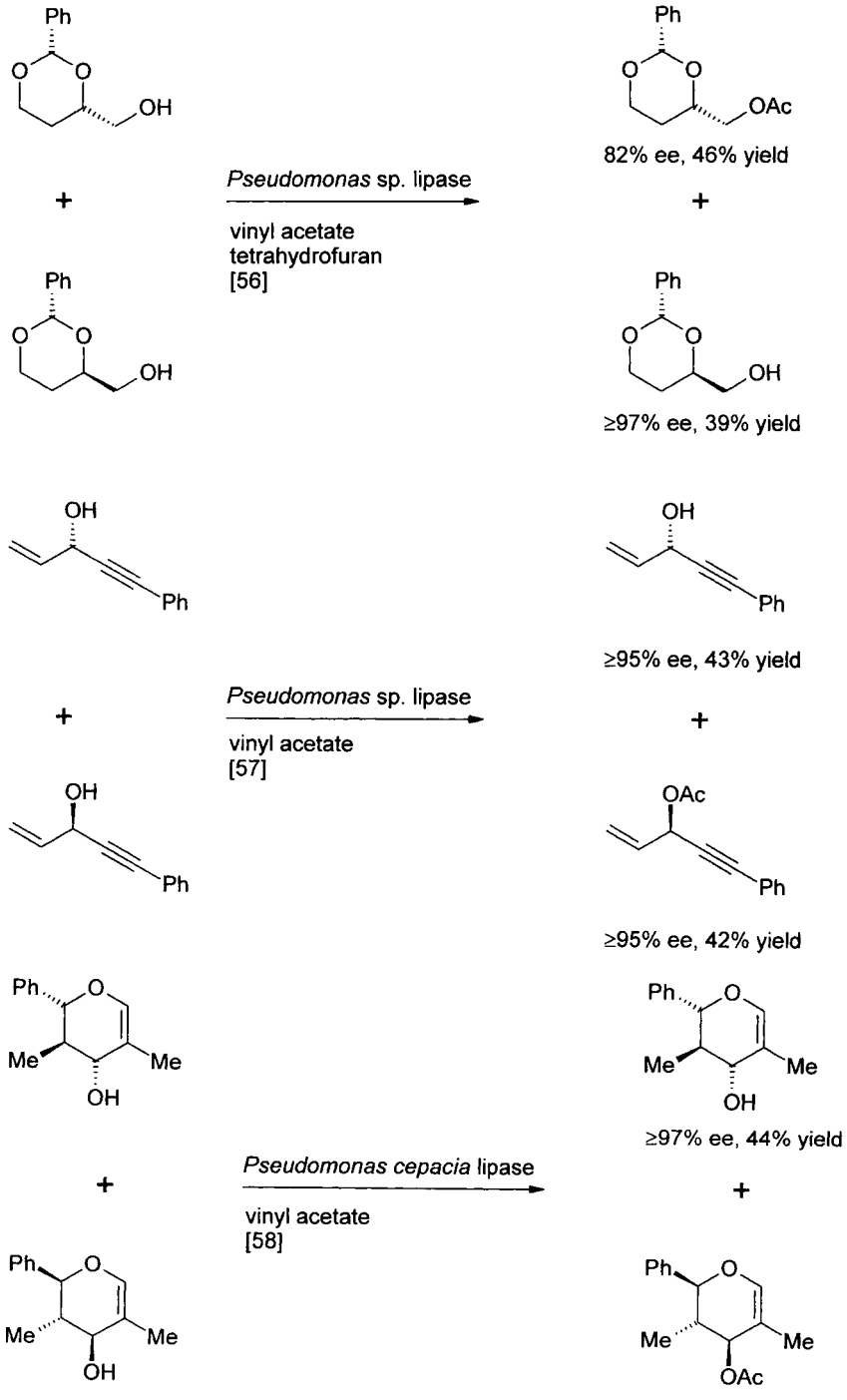
the serine residue in the active site of the enzyme on the carbonyl group of the substrate or reagent with formation of a covalent acyl-enzyme-product complex and its transformation to the free acyl-enzyme and H-X-R^2 [Eq. (1)][63]; reaction of the acyl-enzyme with a nucleophile, as for example water or an alcohol with formation of an acyl-enzyme substrate complex, which reacts with deacylation and formation of another enzyme product complex, which finally gives the free enzyme and the product [Eq. (2)]. The overall equilibrium, the attainment of which is catalyzed by the



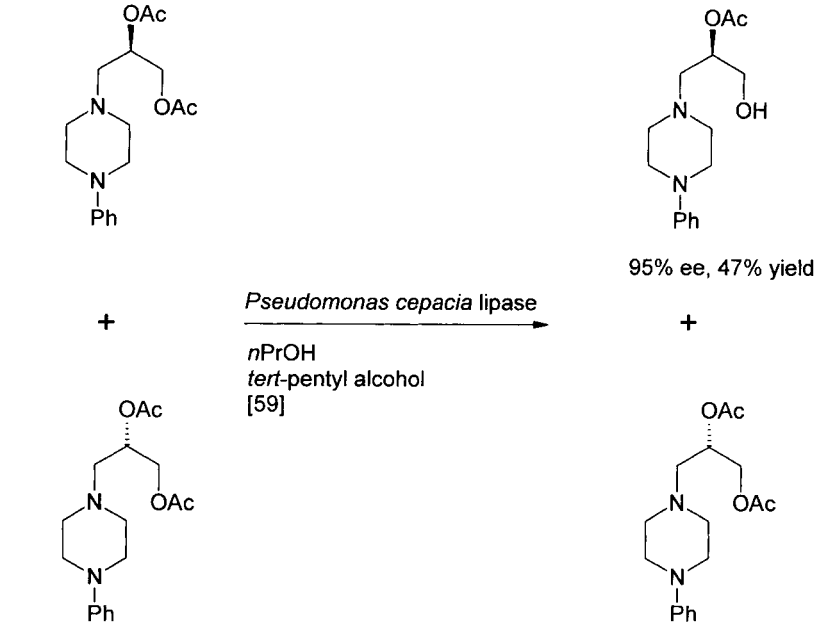
Scheme 11.1-5. Enantiotopos-differentiating hydrolysis of acetates.

enzyme, is depicted in Eq. (3). All steps are in principle reversible. Formation of the acyl-enzyme and its reaction with a nucleophile involves the enzyme-bound tetrahedral intermediates **A** and **B**. In these processes a triad of three amino acids of the active site of the enzyme, Ser, His and Asp(Glu), which are specifically orientated in a three-dimensional way, together with other amino acids is involved. Crucial to the catalytic function of the enzyme are, besides the interplay of the residues of these amino acids, the stabilization of the oxy anion intermediates **A** and **B** and the corresponding transition states through hydrogen bonds provided by amide bonds or other amino acid residues of the active site.

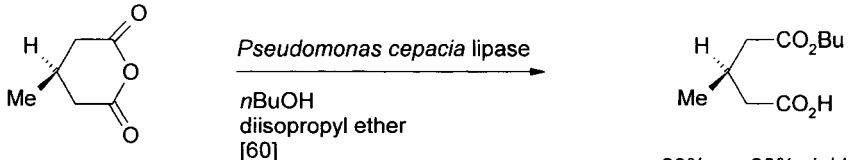
Hydrolysis of C-O bonds of esters and lactones [Eqs. (1) to (3), X = O and YR^3 = OH] is usually carried out at room temperature in aqueous solution or in mixtures of water and either a water-miscible or water-immiscible solvent. Because of the large excess of water, equilibrium usually is mainly if not completely on the side of the

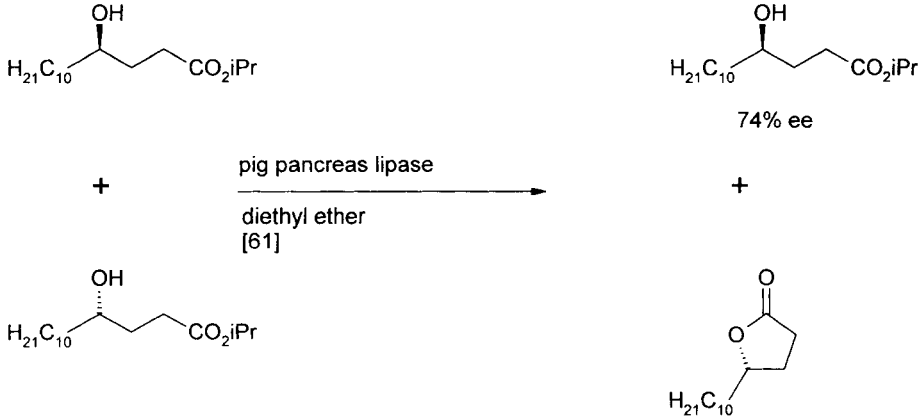


Scheme 11.1-6. Enantiotopos-differentiating transesterification of alcohols.



Scheme 11.1-7. Enantiotopos-differentiating alcoholysis of acetates.





Scheme 11.1-8. Enantiotopos-differentiating lactonization of hydroxy esters.

R^1–CO–X–R^2 + enzyme

⇅

R^1–CO–X–R^2 • enzyme

⇅

R^1–CO–enzyme • H–X–R^2

⇅

R^1–CO–enzyme + H–X–R^2

(Eq.1)

R^1–CO–enzyme + H–Y–R^3

⇅

R^1–CO–enzyme • H–Y–R^3

⇅

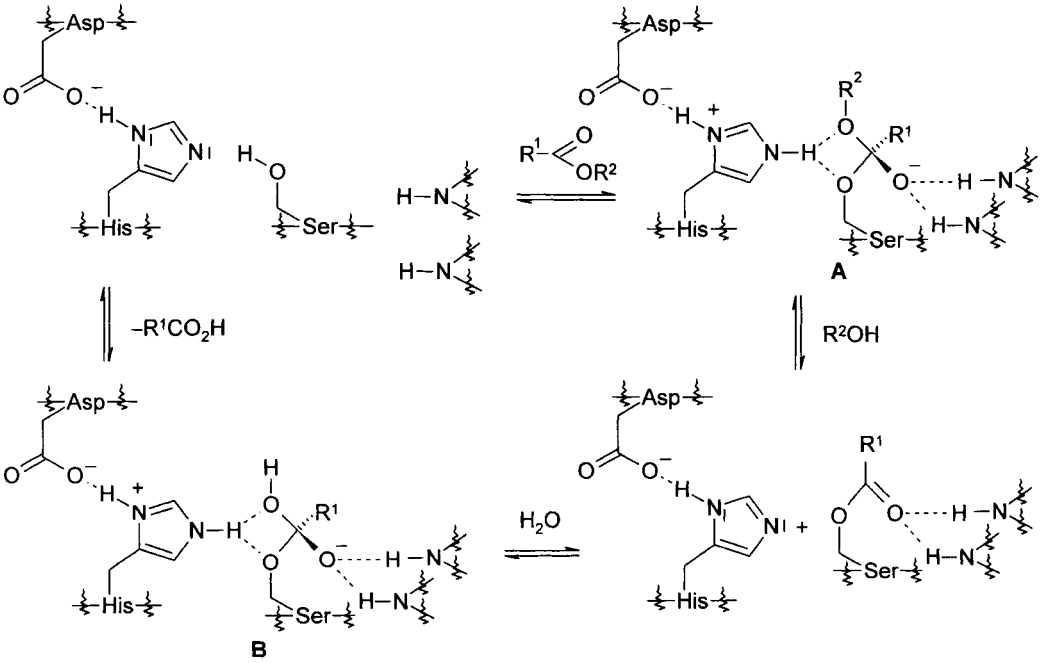
R^1–CO–Y–R^3 • enzyme

⇅

enzyme + R^1–CO–Y–R^3

(Eq. 2)

$$R^1\text{–CO–X–}R^2 + \text{H–Y–}R^3 \rightleftharpoons R^1\text{–CO–Y–}R^3 + \text{H–X–}R^2 \quad \text{(Eq.3)}$$



Scheme 11.1-9. Mechanism of hydrolase-catalyzed reaction.

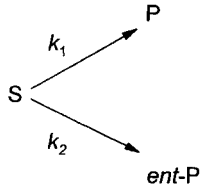
carboxylic acid and the alcohol [Eq. (3), X, Y = O, R^3 = H] and the reaction is practically irreversible. Low solubility of liquid substrates normally presents no problem. In fact lipases are designed by nature to work at the liquid-liquid interface. In the case of crystalline and also liquid substrates of low solubility, solubility may be

enhanced by addition of an organic solvent. Most commonly used cosolvents are alcohols, as for example methanol and *tert*-butanol, and acetonitrile, acetone, tetrahydrofuran, ether, dimethylformamide and dimethyl sulfoxide[9, 28, 30, 34, 36]. The choice of the cosolvent, miscible or immiscible with water, depends on the enzyme. Lipases frequently show higher activity and selectivity in an emulsion of water and *tert*-butyl methyl ether or diethyl ether than in water. At higher concentrations of the organic solvent, the stability and the activity of the enzyme may be very low and the presence of the cosolvent can also alter the enantioselectivity of the enzyme in both directions. Many hydrolases, except lipases, show an interfacial deactivation. In aqueous solution, the parameters which influence the rate of hydrolysis most are the pH value and the temperature. In most cases, hydrolysis is run at room temperature at pH 7.0. However, at lower temperatures the selectivity of hydrolysis may be higher. For an easy recovery of the enzyme from the aqueous solution and for other purposes, it can be immobilized by various techniques[30, 34, 36, 64a]. One of the most popular for laboratory scale synthesis is the covalent immobilization of the enzyme on Eupergit C, a polymer with reactive epoxide groups. However, the nature of the polymer and the method of fixation may greatly influence the stability and activity of the immobilized enzyme. Another very useful technique, especially for synthesis on a larger scale, is the use of a continuous flow membrane reactor[64b]. Recovery of the enzyme from aqueous solution in small- and large-scale batch synthesis can be done by ultrafiltration[64c]. Finally, cross-linked enzyme crystals (CLECs), which are crystals of a pure enzyme cross-linked with glutaraldehyde, can be used for this and other purposes because of their insolubility in water and organic solvents[64e].

Formation of C-O bonds of esters and lactones is accomplished by exploiting the transferase activity of hydrolases in acylation and alcoholysis (transesterification) reactions in organic media of low water content[30, 34, 36]. Especially lipases show a high activity in organic solvents, as for example ethers, hydrocarbons, chlorinated hydrocarbons, vinyl acetate, isopropenyl acetate and ethyl acetate. Lipases are thus by far the most useful hydrolases for asymmetric synthesis in organic media (Sect. 11.1.1.2.1). Subtilisin (Sect. 11.1.1.2.2.), pig liver esterase (Sect. 11.1.1.2.3) and α-chymotrypsin (Sect. 11.1.1.1.2), which also show activity in organic media of low water content, have been applied only to a lesser extent in asymmetric synthesis through C-O bond formation. Lipases, like other hydrolases, are generally not soluble in organic solvents. They rather form suspensions of protein aggregates. Enyzmes are only active if a certain amount of water, which is adsorbed or bound by the enzyme or dissolved in the organic solvent, is present. Organic solvents with a high solubility for water are generally not well suited for a hydrolase-catalyzed transesterification, perhaps because of a dehydration of the enzyme. It is frequently observed that the enantioselectivity of the acylation of an alcohol, catalyzed by a lipase, in an organic solvent is higher than the hydrolysis of the corresponding ester catalyzed by the same lipase in water. To provide for extreme equilibrium positions in acylation reactions of alcohols catalyzed by lipases in organic media, fatty acid trifluoro- or trichloroethyl esters, vinyl esters, oxime esters or carboxylic acid anhydrides have been used with much success. They provide for extreme equilib-

rium positions and practically irreversible reactions. Among these acylation reagents, vinyl esters are the most useful. However, the formation of acetaldehyde in transesterification with vinyl esters may cause a deactivation of the enzyme, most probably because of a reaction with its lysine amino groups[65, 66]. Generally, the lipases used for the formation of C-O bonds under these conditions are crude preparations which may contain only a few percent of the actual lipase. These crude lipases usually also contain other proteins (which may be even other enzymes), additives such as carbohydrates, and salts, which stabilize the enzymes. Usually fair amounts of these crude materials are used as suspension in organic solvents together with the acyl transfer reagent. The water content of the system is in most cases only ill defined, and it can decrease through a competing hydrolase-catalyzed hydrolysis of the acylation reagent[65, 66]. Because of the insolubility of hydrolases in most organic solvents, catalysis is carried out under heterogeneous conditions, which restrict not only the mobility of the enzyme but also of the substrate and products, and can thus cause mass transfer limitations. Immobilization on solid support, addition of hydrated salts, covalent attachment of methoxpoly(ethylene glycol) (MPEG) residues, lyophilization with organic polymers, cross-linking of enzyme crystals, sol-gel entrapment, coating with amphiphilic molecules and entrapment in reverse micelles are the most important techniques that have been shown to improve the performance of the enzyme[36]. Some of these measurements serve to increase the surface area of the enzyme and thus to enhance its activity. Among the various types of hydrolase-catalyzed reactions, those involving the differentiation of enantiotopic ester groups (-CO_2R), acyloxy groups (-OCOR) and hydroxyl groups or the differentiation of the enantiomers of esters of racemic carboxylic acids and racemic alcohols are by far the most important ones. Under non-equilibrium conditions (e.g. hydrolysis in the presence of a large excess of water, alcoholysis with a large excess of an alcohol in organic solvents of low water content, or acylation with a vinyl ester in organic solvents of low water content), the decisive steps of differentiation are as follows. In the case of differentiation of enantiotopic ester groups or enantiomeric esters starting with the enzyme it may be the formation or cleavage of the acyl-enzyme; in the case of enantiotopic acyloxy groups or enantiomeric esters of racemic alcohols starting with the enzyme it may be the formation or break-down of intermediate **A** with generation of the acyl-enzyme; and in the case of enantiotopic hydroxyl groups or the enantiomers of racemic alcohols starting with the acyl enzyme it may be the formation or break-down of intermediate **A** with formation of the enzyme.

Numerous *meso*-configured or otherwise prochiral substrates, preferentially containing enantiotopic methoxycarbonyl groups, have been converted by a pig liver esterase- or lipase-catalyzed enantioselective hydrolysis in water to chiral monoesters (see Sect. 11.1.1.1.1., Tables 11.1-1 to 11.1-4 and Sect. 11.1.1.1.5, Tables 11.1-10 to 11.1-12). In nearly all cases investigated thus far the pig liver esterase-catalyzed hydrolysis of the substrate diester S terminates at the stage of the enantiomeric monoesters P and *ent*-P. In this case, where the products P and *ent*-P are not transformed further, the irreversible enantiotopos-differentiation may be described by the process depicted in Scheme 11.1-10[67-69].

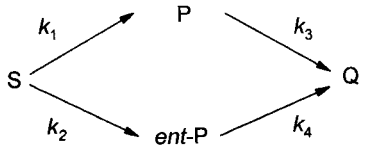


Scheme 11.1-10. Hydrolase catalyzed enantiotopos-differentiating irreversible transformation[67]. S: substrate; P, *ent*-P: enantiomeric products; k_1, k_2: apparent first-order rate constants of the irreversible process; E: selectivity factor; ee: enantiomeric excess.

$$E = \frac{k_1}{k_2} = \frac{[P]}{[ent\text{-}P]} \qquad ee\ (P) = \frac{E-1}{E+1}$$

Thus, in this case the *ee* value of the monoester P (or *ent*-P) is determined by the selectivity of enantiotopos-differentiation. It is not depended on the extent of the hydrolysis but only on the ratio of the two apparent first-order rate constants k_1 and k_2 if one assumes an irreversible reaction and the absence of product inhibition, the former assumption being reasonable because of the large excess of water. In case of an insufficient degree of differentiation, selectivity can be raised only by a suitable temporary or permanent modification of the structure of the substrate S, by choice of another hydrolase, by addition of an organic solvent or by variation of the temperature or pH value, but not by stopping the reaction at various degrees of conversion. In practice the situation is somewhat different in the case of *meso*-configured or otherwise prochiral diacylated diols having enantiotopic acyloxy groups. For these diesters, hydrolysis by pig liver esterase and lipases in water usually does not stop completely at the stage of the enantiomeric monoester P and *ent*-P, but proceeds further – although at a significantly lower rate – to the achiral diol Q (Scheme 11.1-11)[67-69]. Thus enantiotopos-differentiation expressed through the apparent first-order rate constants k_1 and k_2 is accompanied by an enantiomer-differentiation as expressed by the apparent first-order rate constants k_3 and k_4.

In this case the *ee* value of the monoester P (or *ent*-P) depends on the extent of the conversion of the diester S to the monoesters P and *ent*-P and of the conversion of the latter to the achiral diol Q, and thus on all four rate constants. From the fact that a hydrolase usually retains the (*R*)- or (*S*)-group preference of the enantiotopos differentiation in the enantiomer-differentiating hydrolysis, *i. e.* the hydrolysis of the faster formed monoester P to the diol Q is slower than the hydrolysis of the slower formed monoester *ent*-P to the diol Q ($k_1 > k_2$ and $k_4 > k_3$ or vice versa), it follows that the *ee* value of the monoester P (or *ent*-P) can be raised upon carrying the hydrolysis further to the diol Q, at the expense of the yield. This can be advantageously used to raise the *ee* value of the monoester to the point where it can be isolated enantiomerically pure (for practical purposes). The diol can in most cases be converted to the diester. A mathematical model for the prediction of the *ee* value of the monoester and the quantity of the individual products in such a combined enantiotopos- and enantiomer-differentiating hydrolysis, which allows one to find the optimum in regard to the *ee* value and the yield, has been developed on the basis of an irreversible reaction and the absence of product inhibition (Scheme 11.1-11)[8, 67-69]. Required are the kinetic constants α, E_1 and E_2, which can be derived from a determination of



Scheme 11.1-11. Hydrolase-catalyzed enantiotopos- and enantiomer-differentiating irreversible transformations[67–69].

$$\alpha = \frac{k_1}{k_2} \qquad E_1 = \frac{k_3}{k_1 + k_2} \qquad E_2 = \frac{k_4}{k_1 + k_2}$$

$$[P] = \frac{\alpha\,[S_0]}{(\alpha+1)\,(1-E_1)} \left[\left(\frac{[S]}{[S_0]}\right)^{E_1} - \left(\frac{[S]}{[S_0]}\right)\right]$$

$$[ent\text{-}P] = \frac{\alpha\,[S_0]}{(\alpha+1)\,(1+E_2)} \left[\left(\frac{[S]}{[S_0]}\right)^{E_2} - \left(\frac{[S]}{[S_0]}\right)\right]$$

$$[Q] = [S_0] - [S] - [P] - [ent\text{-}P]$$

$$ee\,(P) = \frac{[P] - [ent\text{-}P]}{[P] + [ent\text{-}P]}$$

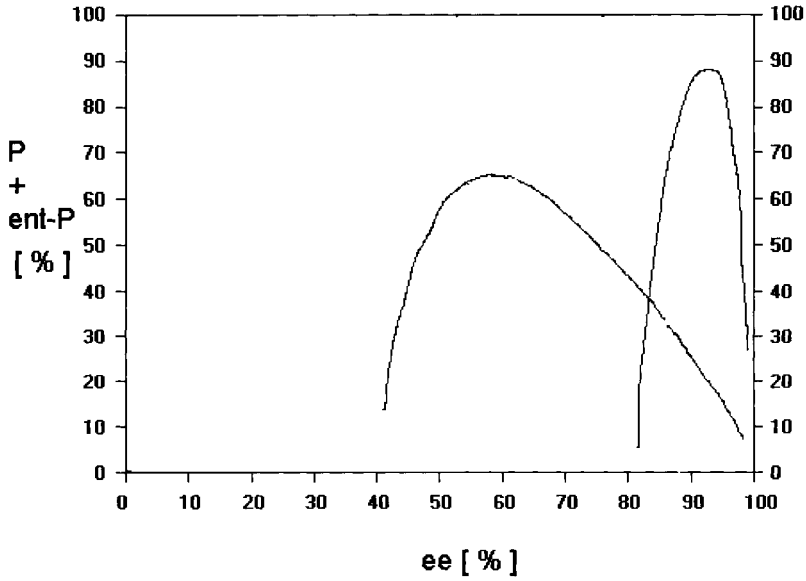
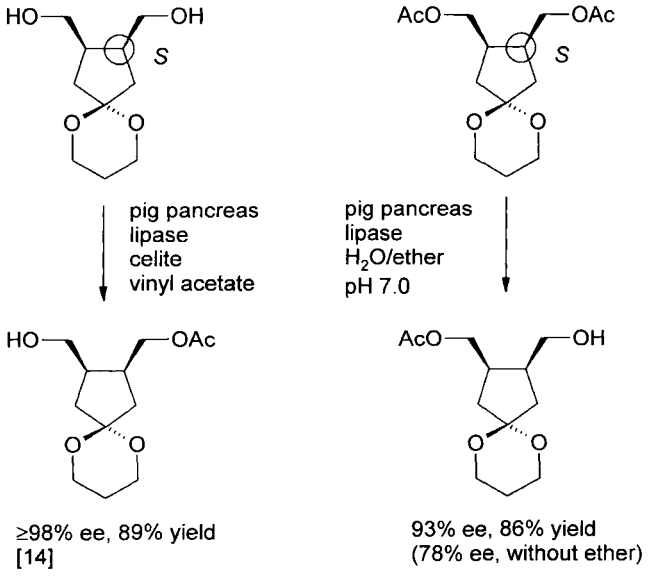


Figure 11.1-1. Dependence of ee value of monoester (P) on yield of monoester in combined enantiotopos- and enantiomer-differentiation with different sets of kinetic parameters.

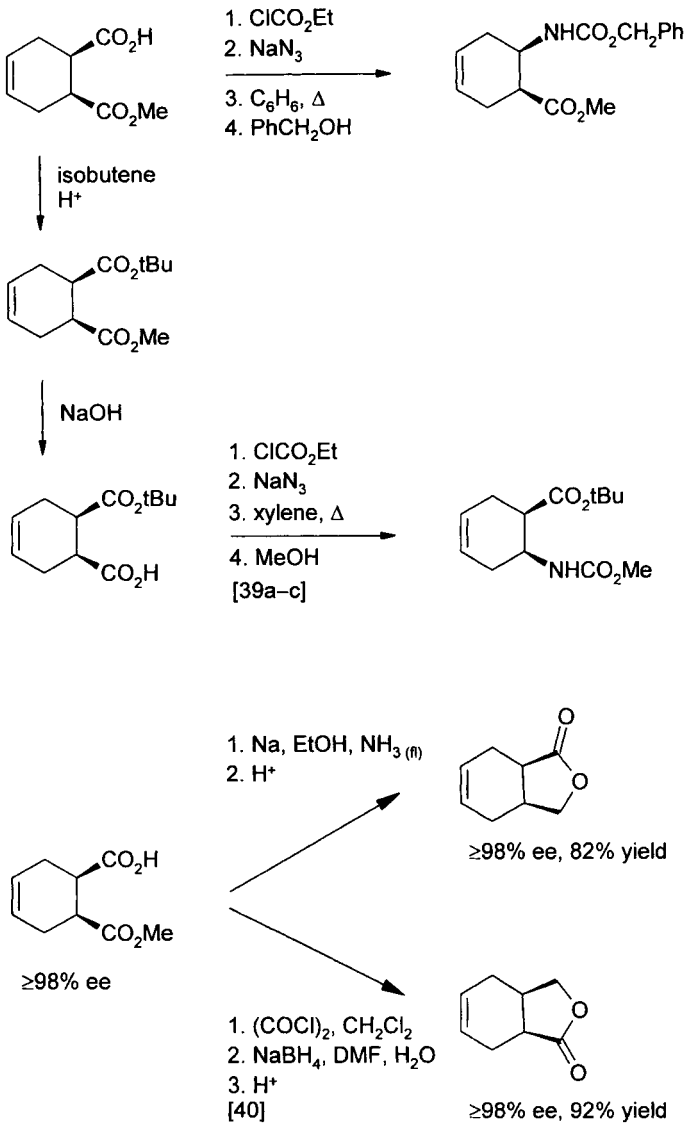
the amounts of S, P and *ent*-P as well as the *ee* values at various stages of the hydrolysis [70]. The *ee* value of the monoester is a function of the conversion, which is generally expressed in curves as schematic depicted in Figure 11.1-1 for two sets of different kinetic constants α, E_1 and E_2 [67-69]. The validity of this has been verified several times [8]. A quite similar situation is encountered in the reverse hydrolysis, *i. e.* the hydrolase-catalyzed acylation of a prochiral diol with, for example, vinyl acetate in an organic solvent of low water content, conditions which render the reaction irreversible, with formation of a chiral monoester. Here the *ee* value of the monoester can also be raised at the expense of the yield through further acylation of the monoester with formation of the achiral diacylated diol. Normally and not surprisingly the hydrolase exhibits in the hydrolysis of the prochiral diacetate and in the acylation of the corresponding prochiral diol the same enantiotopic group recognition despite the fact that chemically different species are involved. This leads to the synthetically favorable situation that generally, through acylation of a prochiral diol in an organic solvent and hydrolysis of the corresponding diacetate in water, both enantiomers of the corresponding monoacetate are accessible with one enzyme (Scheme 11.1-12) [1-36]. The validity of this approach has been demonstrated in numerous cases.

Chiral monoesters, obtained either from a prochiral diol or diester, may be converted by a suitable series of chemoselective transformation to either enantiomer of a given target compound (enantiodivergent synthesis) (Scheme 11.1-13) [10, 40].

Because of the results with numerous prochiral diesters and diols, which have been subjected successfully to hydrolase-catalyzed enantioselective hydrolysis and acylation, respectively, and because of the desire to predict the sense of the asymmetric induction in the conversion of a new substrate, active-site or substrate models have been developed for the hydrolases pig liver esterase [71-73], pig pancreas



Scheme 11.1-12. Synthesis of both enantiomers of a monoacetate through transesterification and hydrolysis with a hydrolase.



Scheme 11.1-13. Synthesis of both eantiomers from a given starting material (enantiodivergent synthesis).

lipase [74], *Pseudomonas cepacia* lipase[75, 76], *Candida rugosa* lipase[76], *Candida antarctica* lipase[77], *Pseudomonas fluorescens* lipase[57, 78], *Pseudomonas aeruginosa* lipase[79], cholesterol esterase[76], subtilisin[80], and α-chymotrypsin[1, 81]. The development of such models is greatly aided by X-ray crystal structure analyses of subtilisin[82], α-chymotrypsin[83], *Candida rugosa* lipase,[84] pig pancreas lipase,[85] *Candida antartica* lipase,[86a, 86b] *Pseudomonas cepacia* lipase[87], and cholesterol esterase[86c]. To a certain extent these models allow for a rationalization of the enantiotopic group and enantiomer preferences observed with the various substrates

and for a prediction in the case of new substrates. Interestingly, X-ray structure analyses show the active site of some lipases in the crystal to be blocked by a helical segment, called a lid or flap. In complexes of those lipases with transition state analogs the lid is opened, permitting access to the active site. Lipases in water usually show a lower activity toward water-soluble substrates than toward water-insoluble, liquid substrates. Thus, interfacial activation of lipases may be caused by a opening of the lid upon contact with a hydrophobic phase[88].

One of the most valuable and much exploited features of hydrolases is their ability not only to differentiate between enantiotopic groups but also to differentiate between enantiomers[1-36]. When, for example, a racemic alcohol or ester is subjected to a hydrolase-catalyzed acylation, alcoholysis or hydrolysis, respectively, a kinetic racemate separation (resolution) can take place, leading, if the process would be completely selective, at the point of 50% conversion, to a mixture of the ester and the corresponding alcohol or acid of opposite configuration. In such a case both the unreacted enantiomer (substrate, S) and the newly formed ester, alcohol or acid (product, P) are enantiomerically pure, and their theoretical yield is 50% based on the racemic substrate. Hydrolase-catalyzed hydrolysis in water, acylation with vinyl acetate in an organic solvent of low water content and alcoholysis (provided that a large excess of alcohol is used) are, all three, practically irreversible, and the efficiency of the racemate separation only depends on the differentiation by the enzyme. When the selectivity of the enantiomer-differentiating hydrolase-catalyzed transformation is insufficient, the enantiomeric purities of the product and of the unchanged substrate can be raised to a certain degree by changing the extent of conversion. Here too a mathematical model for the prediction of the *ee* value of the product and the unreacted substrate as function of the degree of conversion and the yield based on the simple classical homocompetitive model, assuming irreversibility and the absence of product inhibition, has been developed (Scheme 11.1-14)[8, 67-70].

By determining the E value from pairs of experimentally determined *c* and *ee*(P) values or *c*- and *ee*(*ent*-P) values, the *ee* values for the product and the substrate, depending on the degree of conversion, can be calculated and thus the optimum in terms of *ee* value and yield be found[89].Three equations for E, called the enantiomeric ratio, allow one to calculate the inherent enantioselectivity of an enzyme, *i. e.* its ability to differentiate between enantiomers. Thus, E values can advantageously be used to compare the inherent enantioselectivities of different enzymes. E values are calculated by one of the equations of Scheme 11.1-14 on the basis of the determination of the conversion *c* and the enantiomeric excess *ee* of the remaining substrate or of the product. Alternatively, E values may be calculated on the basis of the *ee* values of both the remaining substrate and the product. Since *ee* values are often more accurately measured than conversion, the third equation is preferred[34]. It should be noted, however, that high E values (> 100) are less accurately determined than moderate E values, because of the enantiomeric ratio being a logarithmic function of the enantiomeric excess. Small changes in the measured enantiomeric purities gives large changes in the E values[34, 90]. Figure 11.1-2 indicates how to proceed practically in cases where the enzyme used exhibits only moderate selectiv-

$$S \xrightarrow{k_1} P$$
$$+$$
$$ent\text{-}S \xrightarrow{k_2} ent\text{-}P$$

Scheme 11.1-14. Hydrolase-catalyzed enantiomer-differentiating irreversible transformation[67–69], c: conversion.

$$E = \frac{\ln \frac{[S]}{[S_0]}}{\ln \frac{[ent\text{-}S]}{[ent\text{-}S_0]}}$$

$$E = \frac{\ln[1 - c\,(1 + ee\,(P)]}{\ln[1 - c\,(1 - ee\,(P)]} \text{ (for c <50\%)}; \quad E = \frac{\ln[(1 - c)(1 - ee\,(S)]}{\ln[(1 - c)(1 + ee\,(S)]} \text{ (for c >50\%)}; \quad E = \frac{\ln\left[\frac{1 - ee\,(P)}{1 + (ee(S)/ee(P)}\right]}{\ln\left[\frac{1 + ee\,(S)}{1 + (ee(S)/ee(P)}\right]}$$

$$c = 1 - \frac{[S] + [ent\text{-}S]}{[S_0] + [ent\text{-}S_0]} = \frac{ee\,(S)}{ee(S) + ee(P)}$$

$$ee\,(P) = \frac{[P] - [ent\text{-}P]}{[P] + [ent\text{-}P]} \qquad ee\,(S) = \frac{[ent\text{-}S] - [S]}{[ent\text{-}S] + [S]}$$

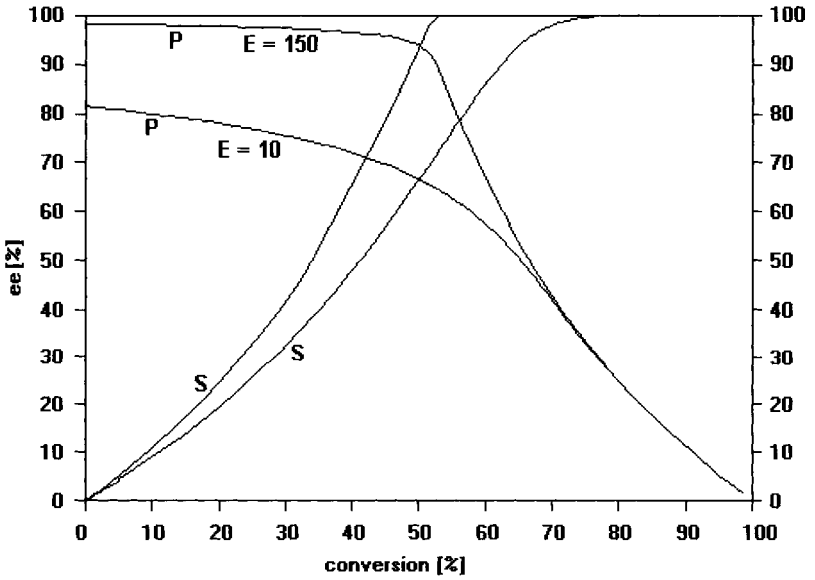
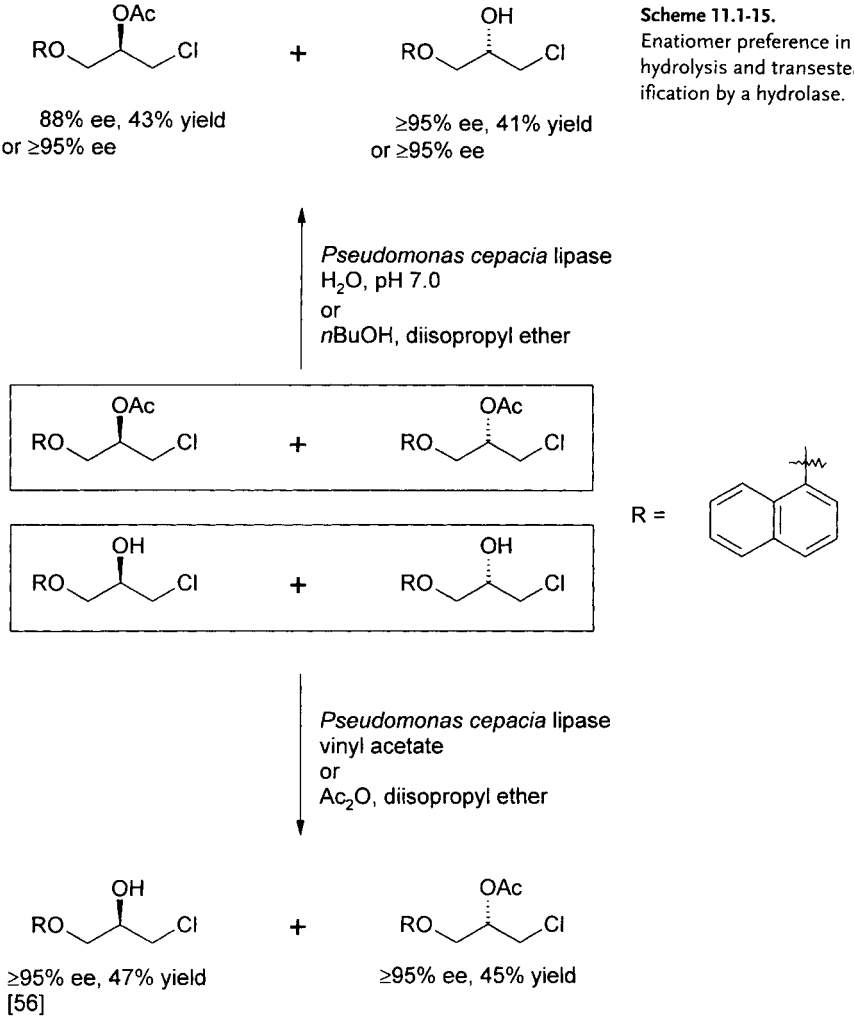


Figure 11.1-2. Dependence of ee value of substrate (S) and product (P) on conversion in kinetic resolution with different E values.



Scheme 11.1-15. Enatiomer preference in hydrolysis and transesterification by a hydrolase.

ity. The product is isolated at the point of < 40% conversion and the substrate is isolated at > 60% conversion. In the case of not very high E values, the substrate but not the product can be obtained enantiomerically pure following this procedure. If the *ee* values of the thus obtained remaining substrate and the product are still not sufficiently high, both compounds may be isolated and subjected to another cycle of hydrolase-catalyzed transformations, either hydrolysis or transesterification, guided by the above principles. Attempts have been made to carry out the second cycle of resolution without the isolation of the product and substrate obtained in the first cycle[91]. In the case of an insufficient selectivity of the resolution of C_2-symmetric substrates, the same approach as for substrates having enantiotopic groups can be applied (see above). For example, the *Pseudomonas cepacia* lipase-catalyzed hydrolysis of racemic *trans*-1,2-diacetoxycyclohexane in a three-phase system composed of

water, *n*-hexane and sodium chloride gave the corresponding (*R*,*R*)-diol with ≥ 99% *ee* in 42% yield and the (*S*,*S*)-diacetate with ≥ 99% *ee* in 38% yield. Critical to the success of this resolution were the following three factors [92]. The enzyme showed a preference for the acetoxy group attached to the (*R*)-center in both the diacetate and in the monoacetate, the monoacetate was completely hydrolyzed to the diol, and an equalization of the rates of the hydrolysis of the diacetate and the monoacetate was be achieved through a favorable partitioning of both between water and *n*-hexane.

A synthetically highly interesting method of converting a racemate completely to one enantiomer is dynamic kinetic resolution, a topic which is dealt with in Sect. 11.1.1.1.5 (Sect. 11.1.2.1.2, Table 11.1-24) on lipases.

Kinetic resolution of alcohols and esters with hydrolases has opened up a new dimension for the synthesis of enantiomerically pure alcohols, esters and carboxylic acids, and in consequence the importance of resolution as a method for the attainment of enantiomerically pure compounds has been increased considerably. Hydrolase-catalyzed resolution is amenable to large-scale production [33–35], as was impressively demonstrated much earlier by the acylase-catalyzed racemate separation of *N*-acyl amino acids (not discussed in this chapter) [64].

In lipase-catalyzed racemate separation of alcohols the same enantiomer preference is usually observed in acylation and hydrolysis (Scheme 11.1-15) [56].

11.1.1
Hydrolysis and Formation of Carboxylic Acid Esters

11.1.1.1
Hydrolysis of Carboxylic Acid Esters

11.1.1.1.1 Pig Liver Esterase

Pig liver esterase (PLE, E.C. 3.1.1.1) is one of the most useful hydrolases for the enantiotopos-differentiating hydrolysis of dicarboxylic diesters and diacetates of diols as exemplified by the hydrolysis of dimethyl *cis*-cyclohex-4-ene-1,2-dicarboxylate [37-42], which yields the corresponding cyclohexenoid monoester in nearly quantitative yield with an *ee* value of ≥ 98% even on a 100 mol scale (Scheme 11.1-1 and Table 11.1-1) [41]. Monoesters of the above type, which are in principle accessible by enzymatic hydrolysis on a large scale, are very useful chiral starting materials for the synthesis of biologically active natural and non-natural compounds [9, 10, 13, 27] including β-amino acids [39a-d]. Up to now approximately 400 substrates for pig liver esterase have been described in the literature. Pig liver esterase, like other hydrolases, does not require a coenzyme, is commercially available, and often combines a low substrate specificity with high enantioselectivity. Pig liver esterase, which is a serine esterase, is isolated as a mixture of isoenzymes composed of the three subunits α (58.2 kDa), β (59.7 kDa) and γ (61.4 kDa), which behave more or less differently in regard to substrate specificity, pH dependence, inhibition or activation by organic solvents or other compounds, and enantioselectivity [93-96]. Commercially available are the natural isoenzyme mixture and several isoenzyme mixtures, enriched in one isoenzyme. These enzyme preparations may contain other proteins

and perhaps even other hydrolases. However, despite this variability in composition, the pig liver isoenzyme mixture has been applied with high success in almost all the cases reported. Even a rather crude acetone extract of pig liver, called pig liver acetone powder (PLAP), was successfully applied to enantioselective hydrolysis. The cloning, functional expression and characterization of recombinant pig liver esterase has been described[97a]. The recombinant pig liver esterase prepared by this method seems to be a single isoenzyme. It was reported that recombinant pig liver esterase, in the kinetic resolution of (1-phenyl-2-butyl)-acetate, shows a much higher selectivity than the isoenzyme mixture[97b]. Pig liver esterase frequently exhibits a reversal in enantiotopic selectivity such as changing from a (*R*)-center to a (*S*)-center ester group preference of hydrolysis within a series of structurally closely related diester substrates. An active-site model for predicting the sense of the enantiotopos-differentiation, which accounts for this reversal, has been proposed[71-73]. Usually the best results are achieved with the dimethyl esters of dicarboxylic acids and with the diacetates of diols. Frequently the enantioselectivity of the hydrolysis and the yield of the monoester may be raised by changing the achiral alcohol component of the ester from methanol to ethanol or isopropanol. In the case of dicarboxylic diesters as substrates, pig liver esterase-catalyzed hydrolysis usually stops completely at the stage of the monoester formed. Further hydrolysis of the monoester to the dicarboxylic acid is in almost all cases thus far investigated extremely slow. This has been attributed to the presence of a charged group (carboxylate) in the molecule. In the case of the diacetates of diols, the rate difference is usually not so great. This, however, in the case of a moderate selectivity of the enantiotopos-differentiating hydrolysis, can be used to enhance the *ee* value of the monoacetate via a subsequent enantiomer-differentiating hydrolysis if the same stereochemical preference is maintained for the enantiomeric monoacetates; *i. e.*, the faster formed enantiomeric monoacetate is more slowly hydrolyzed to the achiral diol, which is usually observed (Scheme 11.1-11). A mathematical model for the prediction of the *ee* value of the monoacetate in such a combined enantiotopos- and enantiomer-differentiating hydrolyses, which allows one to find the optimum in regard to the *ee* value and yield, has been developed for pig liver esterase-catalyzed hydrolyses[67–69]. Pig liver esterase-catalyzed hydrolyses are generally carried out in aqueous phosphate buffer solution at pH 6-8 at room temperature. Equilibrium is under such conditions well on the side of the hydrolysis products because of the large excess of water. Normally, in the case of liquid substrates, low water solubility represents no problem. In the case of crystalline and liquid substrates of low solubility in water, up to 20% of organic cosolvents such as acetone, methanol, *tert*-butanol or dimethyl sulfoxide may be added[98–100]. It should be noted, however, that the enantioselectivity and the rate of the hydrolysis as well as the yield of the product may be influenced in either direction by organic cosolvents. Since pig liver esterase is a mixture of isoenzymes, it has been speculated that in the presence of organic cosolvents one or more isoenzymes might be deactivated. Organic cosolvents can in some cases be advantageously used to enhance the *ee* value of the chiral monoester or monoacetate. Pig liver esterase can be recovered from the aqueous solution by ultrafiltration[41] or by immobilizing the enzyme covalently on oxirane-activated acrylic beads (Eupergit

Table 11.1-1. Pig liver esterase-catalyzed enantiotopos-differentiating hydrolysis of prochiral cyclic dicarboxylic acid diesters in aqueous solution.

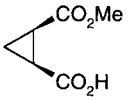
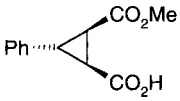
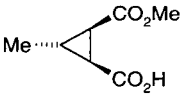
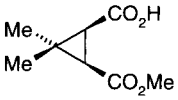
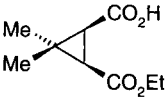
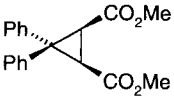
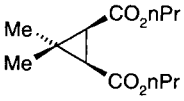
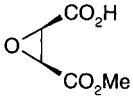
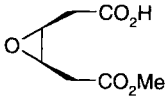
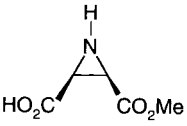
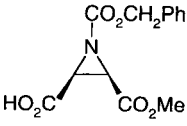
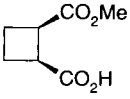
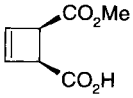
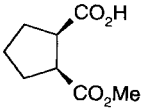
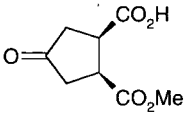
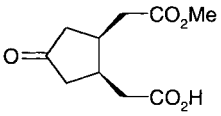
 100% ee, 99% yield	**1** [1–4, 5]	 88% ee, 99% yield	**2** [6, 7]
 91% ee, 90% yield	**3** [6, 7]	 74% ee, 95% yield	**4** [1, 3, 6]
 45% ee, 80% yield	**5** [6]	 no hydrolysis	**6** [6]
 no hydrolysis	**7** [6]	 31% ee, 65% yield	**8** [4, 8]
 99% ee, 90% yield	**9** [9]	 92% ee	**10** [10]
 38% ee	**11** [10]	 94% ee, 98% yield	**12** [1–4, 7]
 86% ee, 96% yield	**13** [11]	 9%, 80% yield	**14** [1, 2, 4, 7]

Table 11.1-1. (cont.).



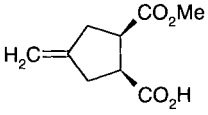
15 [12, 7]

72% ee, 83% yield
82% ee, 85% yield, 10% MeOH
60% ee, 78% yield, 10% acetone



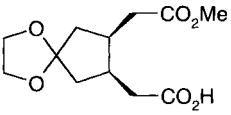
16 [12, 13]

88% ee, 85% yield



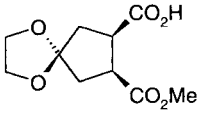
17 [12, 14]

22% ee, 63% yield



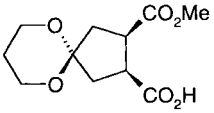
18 [13]

90% ee, 99% yield



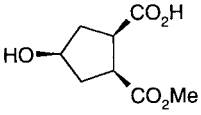
19 [12]

0% ee, 92% yield
(isolated as ketone)



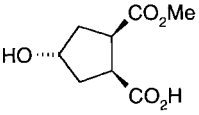
20 [12]

48% ee, 87% yield
(isolated as ketone)



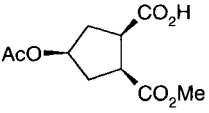
21 [12]

80% ee, 76% yield



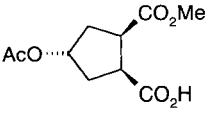
22 [12]

22% ee, 80% yield



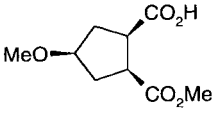
23 [12]

52% ee, 76% yield



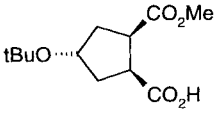
24 [12]

28% ee, 91% yield



25 [12]

58% ee, 88% yield



26 [12]

84% ee, 76% yield

Table 11.1-1. (cont.).

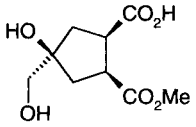
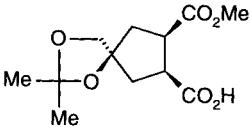
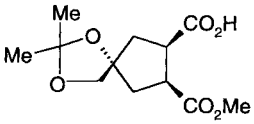
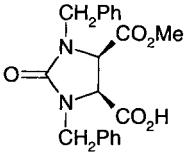
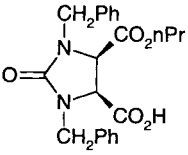
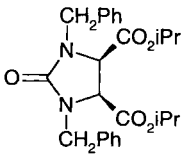
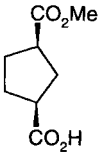
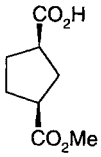
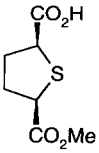
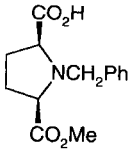
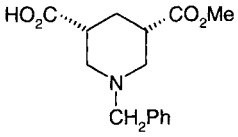
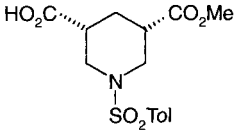
 73% ee	**27** [14]	 64% ee	**28** [14]
 6% ee	**29** [14]	 38% ee, 71% yield	**30** [15]
 75% ee, 85% yield	**31** [15]	 no hydrolysis	**32** [15]
 34% ee, 82% yield	**33** [15]	 42% ee, 98% yield	**34** [16]
 46% ee, 83% yield	**35** [16]	 17% ee, 85% yield 100% ee, 39% yield, 25% DMSO 100% ee, 10% MeOH 61% ee, 10% DMSO 39% ee, 10% MeCN	**36** [17, 18, 19]

Table 11.1-1. (cont.).



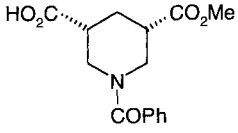
37 [20]

44 % ee, 28 % conversion
27 % ee, 96 % conversion



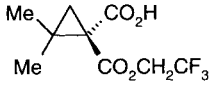
38 [20]

14 % ee, 31 % conversion
11 % ee, 88 % conversion



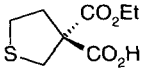
39 [20]

55 % ee, 16 % conversion
34 % ee, 33 % conversion



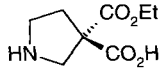
40 [21]

≥95 % ee, 62 % yield



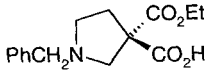
41 [22]

6 % ee, 83 % yield



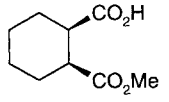
42 [22]

20 % ee, 83 % yield



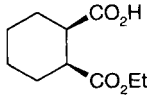
43 [22]

10 % ee, 63 % yield



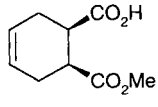
44 [1, 2, 7, 23, 24]

80 % ee, 99 % yield



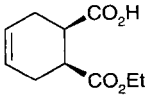
45 [25]

0 % ee



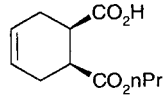
46 [1, 23, 24, 26, 27, 28, 29]

≥98 % ee, 99 % yield



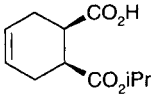
47 [30]

27 % ee, 67 % yield



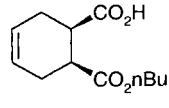
48 [30]

25 % ee, 68 % yield



49 [30]

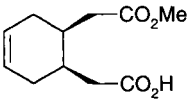
2 % ee, 5 % yield



50 [30]

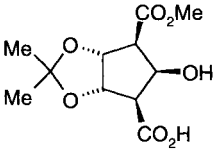
13 % ee, 18 % yield

Table 11.1-1. (cont.).



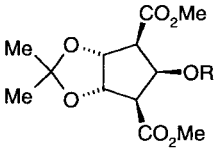
68 % ee, 84 % yield

51 [12, 31]



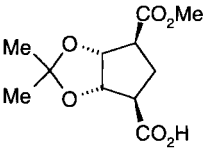
≥95 % ee, 42 % yield

52 [32, 33]



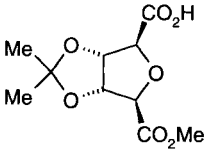
R = *t*Bu no hydrolysis
R = H 95 % ee

53 [34]



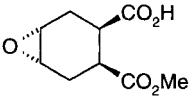
60 % ee

54 [35]



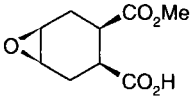
72 % ee, 86 % yield

55 [36]



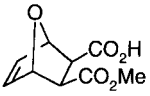
96 % ee, 72 % yield
(isolated as derivative)

56 [37]



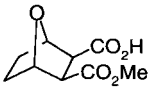
no hydrolysis

57 [9]



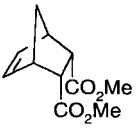
75 % ee, 86 % yield

58 [38, 39]



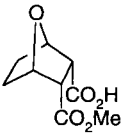
98 % ee, 82 % yield

59 [38]



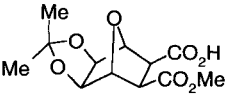
no hydrolysis

60 [38]



64 % ee, 87 % yield

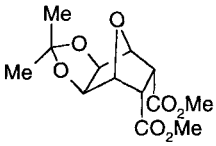
61 [38]



0 % ee, 10 % yield

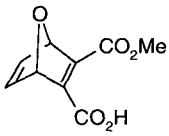
62 [40]

Table 11.1-1. (cont.).



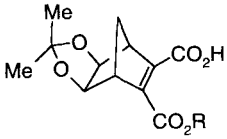
63 [41]

no hydrolysis



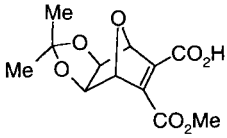
64 [42]

36 % ee, 77 % yield



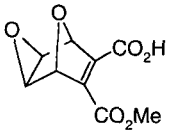
65 [43, 44]

R = Me 80 % ee, 10 % yield
R = Et 100 % ee, 37 % yield
R = *n*Pr 45 % ee, 15 % yield
R = iPr 39 % ee, 22 % yield
R = *n*Bu 73 % ee, 4 % yield



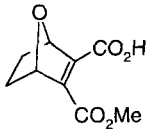
66 [40, 45]

77 % ee, 96 % yield



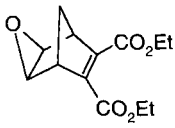
67 [40, 45, 46]

77 % ee, 100 % yield



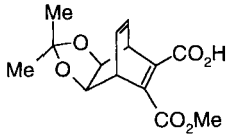
68 [47]

≥95 % ee, 99 % yield



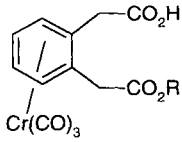
69 [48]

65 % ee, 95 % yield



70 [49]

88 % ee



71 [50, 51]

R = Me 94 % ee
R = Et 99 % ee

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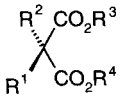
C)[101], BrCN-Sepharose[101], silica gel[102], or in a hollow fiber ultrafiltration membrane[42]. The Eupergit C immobilized pig liver esterase retains 68% of the specific activity of the soluble enzyme. It is easily removed by filtration from the reaction mixture and can be reused several times when stored at 7 °C. In large-scale experiments with pig liver esterase in aqueous solution the enzyme can be stabilized, if necessary, by the addition of inexpensive bovine serum albumin[41]. For a determination of the *ee* value of the monoester, different methods can be used:

addition of an enantiomerically pure chiral amine as for example ephedrine[40] or α-phenylethylamine[103] and ^{1}H NMR spectroscopy of the diastereomeric salts formed thereby, conversion of the dicarboxylic acid monoester to the α-phenylethylamide and analysis by ^{1}H NMR spectroscopy or HPLC[104], or conversion to the *tert*-butyl ester and ^{1}H NMR spectroscopy in the presence of a chiral shift reagent[105]. Determination of the *ee* value of monoacetates has been carried out for example through conversion to the Mosher-ester and analysis by ^{1}H NMR spectroscopy in the presence or absence of a shift reagent or by HPLC[106], or more directly either by ^{1}H NMR spectroscopy in the presence of a chiral shift reagent or by HPLC and GC on chiral columns[107].

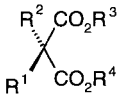
Cyclic dicarboxylic acid diesters, which bear enantiotopic ester groups, are substrates par excellence for a pig liver esterase-catalyzed hydrolysis under formation of the corresponding monoesters (**1–71**) (Table 11.1-1). The examples listed in Table 11.1-1 are a good demonstration of the scope and limitation of pig liver esterase-catalyzed hydrolysis and illustrate general trends. The enantioselectivity and the yield can be influenced to a certain extent by the structure of the alcohol moiety of the diester as exemplified by the heterocyclic monomethyl and monopropyl esters **30** and **31**. The corresponding isopropyl ester **32** is not a substrate for pig liver esterase. Extreme examples for the influence of the alcohol moiety are the cyclohexanoid methyl ester **44**, which is formed with an *ee* value of 80% and the ethyl ester **45**, which is produced as racemate. Usually the dimethyl esters are the best substrates. This trend, however, is not general. In the series of the cyclohexenoid monoesters **46–50**, the methyl ester **46** is the one formed with the highest *ee* value, and, in the series of the bicyclic monoesters **65** having a norbornene skeleton, it is the ethyl ester which has the highest *ee* value. However, the methyl ester **65** (R = Me) is formed much faster and is obtained in higher yield. Addition of an organic cosolvent such as dimethyl sulfoxide or methanol can lead to the monoester with a higher *ee* value. This has been impressively demonstrated in the case of the benzyl-protected heterocyclic monoesters **36**. A generalization of the effects of organic solvents upon the enantioselectivity of the PLE-catalyzed hydrolysis of diesters is difficult. As exemplified by the series of cyclopentanoid monoesters **14**, **15**, **17**, **19–29**, seemingly small structural changes may invert the enantiotopic recognition through the enzyme. This can be advantageously used in certain cases to raise the *ee* value of a given target monoester by a suitable substrate modification. Branching in the α-position of the ester group is no prerequisite for high enantioselectivity. The monoesters **16** and **18**, the ester groups of which are separated by a methylene group from the ring, are obtained with comparable enantioselectivities. Interestingly, enantiotopic recognition is reversed in the series of diesters corresponding to the monoesters **15** and **16** but not with the diesters corresponding to the monoester **8**, **9**, **17** and **18**. This seemingly unpredictable behavior of pig liver esterase may at a first glance detract from its use in asymmetric synthesis. However, successful attempts have been made to rationalize this observation as well as the sense of asymmetric induction observed with the various substrates within an active-site model of the enzyme. Furthermore, one should bear in mind the experimental simplicity of a pig liver esterase-catalyzed hydrolysis and the synthetic advantages gained if the diester

Table 11.1-2. Pig liver esterase-catalyzed enantiotopos-differentiating hydrolysis of prochiral acyclic dicarboxylic acid diesters in aqueous solution.



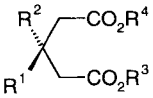
	R^1	R^2	R^3	R^4	ee (%)	yield (%)	Ref.
1	CH_3	C_2H_5	CH_3	H	73[a]	90–98	[1]
2	CH_3	n-C_3H_7	CH_3	H	52[a]	90–98	[1, 2]
3	CH_3	i-C_3H_7	CH_3	H	19[b]	87	[3]
4	CH_3	n-C_4H_9	CH_3	H	58[a]	90–98	[1]
5	CH_3	n-C_5H_{11}	H	CH_3	46[a]	90–98	[1]
6	CH_3	n-C_6H_{11}	H	CH_3	87[a]	90–98	[1]
7	CH_3	n-C_7H_{15}	H	CH_3	88[a]	90–98	[1]
8	CH_3	$CH_2C_6H_5$	H	CH_3	16[a]	90–98	[1]
9	CH_3	F	CH_3	H	16[b]	61	[4]
10	CH_3	p-$CH_3OC_6H_4CH_2$	H	CH_3	82[c]	85–100	[5]
11	CH_3	3,4-CH_3O-$C_6H_3CH_2$	H	CH_3	93[c]	85–100	[5]
12	CH_3	p-$CH_3C_6H_4$	CH_3	H	96	–	[6]
13	CH_3	$HOCH_2$	CH_3	H	6	37	[7]
14	CH_3	CH_3OCH_2	CH_3	H	21	86	[7]
15	CH_3	$C_6H_5CH_2OCH_2$	H	CH_3	67	90	[3, 8]
16	CH_3	$(CH_3)_3COCH_2$	H	CH_3	96	90	[3, 9]
17	CH_3	$(CH_3)_3C(CH_3)_2SiOCH_2$	H	CH_3	95	49	[3]
18	CH_3	$BrCH_2$	CH_3	H	46[b]	–	[3]
19	CH_3	$C_6H_5(CH_2)_2$	CH_3	H	84[b]	76	[3]
20	CH_3	$C_6H_5(CH_2)_3$	CH_3	H	87[b]	81	[3]
21	CH_3	$(CH_3)_3C(CH_2)_2$	CH_3	H	96[b]	95	[3]
22	CH_3	OH	CH_3		46[b]	46	[1]
23	C_2H_5	C_6H_5	CH_3	H	84[b]	–	[10]
24	i-C_3H_7	$C_6H_5(CH_2)_2$	CH_3	H	no hydrolysis	–	[3]
25	CH_3	$(CH_3)_3SiCH_2$	H	CH_3	88[b]	86	[11]
25	CH_3	$(CH_3)_3SiCH_2$	H	CH_3	98[b,c]	95	[11]
26	CH_3	C_2H_5	C_2H_5	H	15[a]	90–98	[1]
26	CH_3	C_2H_5	C_2H_5	H	20	–	[12]
27	CH_3	n-C_3H_7	C_2H_5	H	10[a]	90–98	[1]
27	CH_3	n-C_3H_7	CH_5	H	8	–	[12]
28	CH_3	n-C_4H_9	C_2H_5	H	25[a]	90–98	[1]
28	CH_3	n-C_4H_9	C_2H_5	H	38	–	[12]
29	CH_3	n-C_5H_{11}	H	C_2H_5	10[a]	90–98	[1]
30	CH_3	n-C_8H_{17}	C_2H_5	H	5[a]	90–98	[1]
31	CH_3	C_6H_5	H	CH_3	81	90	[1]
32	CH_3	C_6H_5	C_2H_5	H	86[b]	–	[1]
33	CH_3	m-$CH_3C_6H_4$	H	CH_3	92	100	[1]
34	CH_3	p-$CH_3C_6H_4$	H	CH_3	82	92	[1]
35	CH_3	p-$C_2H_5C_6H_4$	H	CH_3	78	100	[1]
36	CH_3	p-$iC_3H_7C_6H_4$	H	CH_3	97	96	[1]
37	CH_3	p-$tC_4H_9C_6H_4$	H	CH_3	97	83	[13]
38	CH_3	CH_3CONH	C_2H_5	H	–[b]	81	[14]
39	H	$(CH_3)_3SiCH_2$	C_2H_5	H	10[b]	92	[11]
40	CH_3	$(CH_3)_3SiCH_2$	C_2H_5	H	80[b]	70	[11]
41	O-NO_2-C_6H_4O	CH_3	C_2H_5	H	69	66	[15]

Table 11.1-2. (cont.).



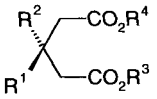
	R^1	R^2	R^3	R^4	ee (%)	yield (%)	Ref.
42	2-NO_2-4-CH_3O-C_6H_3O	CH_3	CH_3	H	80	78	[16]
43	2-NO_2-4-Cl-C_6H_3O	CH_3	C_2H_5	H	78	66	[16]
44	2-NO_2-5-F-C_6H_3O	CH_3	CH_3	H	69	84	[16]
45	2,5-NO_2-C_6H_3O	CH_3	CH_3	H	79	77	[16]
46	$(CH_3)_3C$	H		H	CH_3	89 87	[17]
47	$((CH_3)_2C_6H_5)C$	H		H	C_2H_5	94 70	[17]
48	1-CH_3-*c*-C_6H_{10}	H		H	C_2H_5	96 45	[17]
49	CH_3	*p*-$CH_3OC_6H_4$		H	C_2H_5	97 98	[18]
50	H	(*E*)-$HOCH_2CH{=}CH{-}CH_2$		CH_3	H	19 99	[19]
51	H	(*E*)-$THPOCH_2CH{=}CH{-}CH_2$		CH_3	H	74 95	[19]
52	H	C_6H_5		CH_3	H	88 97	[19]
53	H	C_6H_5		C_2H_5	H	92 99	[19]

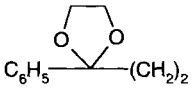
a In the presence of 25% dimethylsulfoxide
b Absolute configuration not determined
c In the presence of 50% dimethylsulfoxide

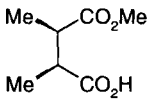


	R^1	R^2	R^3	R^4	ee (%)	yield (%)	Ref.
54	CH_3	H	H	CH_3	90	86	[20, 21]
54	CH_3	H	H	CH_3	79	94	[22]
54	CH_3	H	H	CH_3	92[d]	–	[22]
55	C_2H_5	H	H	CH_3	50	67	[22]
56	*n*-C_3H_7	H	H	CH_3	25	78	[22]
57	*i*-C_3H_7	H	CH_3	H	38	98	[22]
58	*c*-C_6H_{11}	H	H	CH_3	17	95	[22]
59	C_6H_5	H	CH_3	H	42	98	[22]
60	H	*p*-FC_6H_4	H	CH_3	95	86	[23]
61	$C_6H_5CH_2$	H	CH_3	H	54	95	[22]
61	$C_6H_5CH_2$	H	CH_3	H	81[d, e]	–	[22]
61	$C_6H_5CH_2$	H	CH_3	H	73	98	[22]
62	$C_6H_5(CH_2)_2$	H	CH_3	H	44	98	[24]
63	$C_6H_5(CH_2)_3$	H	CH_3	H	88	97	[24]
64	(*E*)–$C_6H_5CH{=}CH{-}CH_2$	H	CH_3	H	88	95	[24]
65	$C_6H_5CH_2O(CH_2)_2$	H	CH_3	H	54	100	[24]
66	(*E*)–$HOCH_2{-}CH{=}CH{-}CH_2$	H	CH_3	H	18	100	[24]
67	(*E*)–$THPOCH_2{-}CH{=}CH{-}CH_2$	H	CH_3	H	74	95	[24]
68	(*E*)–$C_6H_5{-}CH{=}CH$	H	CH_3	H	93	100	[24]
69	NH_2	H	H	CH_3	41	94	[25]
70	CH_3CONH	H	H	CH_3	93	81	[25]
71	C_2H_5CONH	H	H	CH_3	6	50	[25]
72	*n*-C_3H_7CONH	H	H	CH_3	15	52	[25]

Table 11.1-2. (cont.).

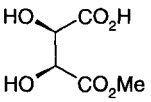


	R^1	R^2	R^3	R^4	ee (%)	yield (%)	Ref.
73	n-C_4H_9CONH	H	CH_3	H	2	48	[25]
74	i-C_3H_7CCONH	H	CH_3	H	54	55	[25]
75	$(CH_3)_3CCONH$	H	CH_3	H	93	50	[25]
76	c-$C_6H_{11}CONH$	H	CH_3	H	79	52	[25]
77	$CH_3CH{=}CH{-}CONH$	H	CH_3	H	100	60	[25]
78	C_6H_5CONH	H	CH_3	H	72	59	[25]
79	$CH_2{=}CHCONH$	H	H	CH_3	8	50	[25]
80	C_6H_5CONH	H	CH_3	H	20	60	[25]
81	C_2H_5CONH	H	CH_3	H	40	70	[25]
82	$(CH_3)_3COCONH$	H	CH_3	H	53	93	[25]
83	$C_6H_5CH_2OCONH$	H	CH_3	H	93	93	[25]
84	$C_6H_5CH_2NH$	H	CH_3	H	33	58	[25]
85	HO	H	CH_3	H	15	100	[18, 26–31, 32]
86	HO	CH_3	CH_3	H	99	62	[21]
87	$CO_2C_2H_5$	OH	C_2H_5	H	90	–	[40]
88	$C_6H_5CH_2O$	H	CH_3	H	40	87	[27, 32]
89	CH_3OCH_2O	H	CH_3	H	14	100	[28]
90	C_6H_5COO	H	CH_3	H	12	78	[28]
91	CH_3COO	H	H	CH_3	90	38	[31]
92	$CH_3O(CH_2)_2OCH_2O$	H	H	CH_3	39	92	[27, 32]
	H	SCH_2-p-$CH_3OC_6H_4$	H	C_2H_5	71	81	[33]
93		H	H	C_2H_5	≥92	–	[34]



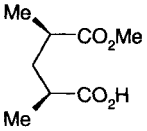
94 [20]

18% ee, 94% yield



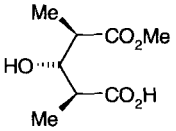
95 [20]

48% ee, 92% yield



96 [20, 35, 36]

64% ee, 85% yield

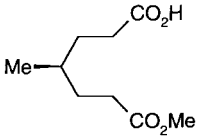


97 [20]

98% ee, 95% yield

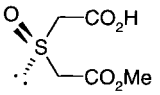
d In the presence of 20% methanol; **e** At −10 °C

Table 11.1-2. (cont.).



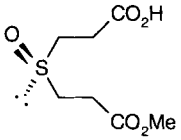
98 [20]

10% ee, 96% yield



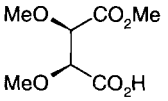
99 [37]

79% ee, 70% yield



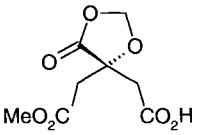
100 [38]

82% ee



101 [39]

90% ee, 90% yield (20% MeOH)



102 [40]

90–92% ee, 80–85% yield

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40 R. Chenevert, B. Ngatcha, R. Tchedam, S. Yannick, D. Goupil, *Tetrahedron: Asymmetry* **1998**, *9*, 4325.

in question is a substrate. Cyclic diesters, the ester groups of which are not in the 1,2-position, seem to be less appropriate substrates for pig liver esterase (**33–39** and **41–43**). Finally, it seems noteworthy that transition metal complexes containing enantiotopic esters groups are also amenable to a highly selective pig liver esterase-catalyzed hydrolysis (**71**). Cyclic monoesters of Table 11.1-1, which can be obtained with other hydrolases as such or of opposite configuration, are contained in Tables 11.1-7 and 11.1-12.

For synthetic and mechanistic reasons, a large number and variety of prochiral malonates have been subjected to pig liver esterase-catalyzed hydrolysis with formation of chiral malonates (**1–53**) (Table 11.1-2). Hydrolysis of dimethyl or diethyl malonates bearing a methyl group and another small alkyl or functionalized alkyl group leads preferentially to the monoester with the (*S*)-configuration. Upon an increase in the size of the second group, enantiotopic recognition is inverted and the monoester with the (*R*)-configuration is formed. Hydrolysis of dimethyl hydroxymethyl methyl malonate provides an excellent example of the strategy to enhance the enantioselectivity of pig liver esterase by the introduction of a protecting group on the substrate. While the parent compound itself yields the corresponding (*S*)-configured monoester **13** with an *ee* value of only 6%, the introduction of a *tert*-butyl or *tert*-butyldimethylsilyl protecting group allows the isolation of the corresponding (*R*)-configured monoesters (**16** and **17**) with *ee* values of 96% and 95%, respectively.

An equally large number and variety of prochiral glutarates have been subjected to pig liver esterase-catalyzed hydrolysis with formation chiral glutarates (**54–93**) (Table 11.1-2). Among the synthetically most useful glutarates are the 3-amino-glutarates. The parent compound methyl amino glutarate **69** itself is obtained only with an *ee* value of 41%. The introduction of an amino protecting group improves the enantioselectivity of the hydrolysis of the corresponding diester dramatically. Pig liver esterase-catalyzed hydrolysis delivers methyl *N*-acetylamino glutarate **70** of (*S*)-configuration with an *ee* value of 93% and methyl *N*-crotonylamino glutarate **77** of the opposite (*R*)-configuration with an *ee* value of 100%. Thus, both enantiomers of methyl amino glutarate are accessible with one enzyme by a synthetically simple substrate modification.

To a limited extent and with only moderate success, *meso*-configured glutarates and succinates have been subjected to pig liver esterase-catalyzed hydrolysis with

Table 11.1-3. Pig liver esterase-catalyzed enantiotopos-differentiating hydrolysis of prochiral cyclic diol diacetates in aqueous solution.

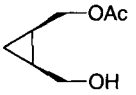
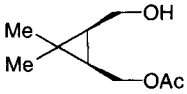
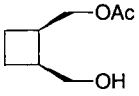
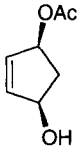
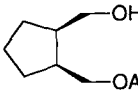
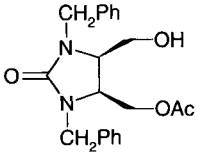
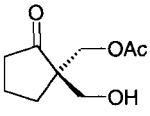
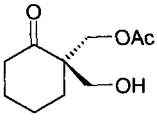
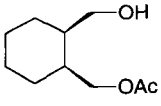
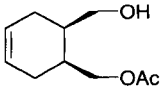
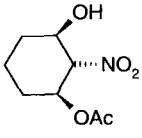
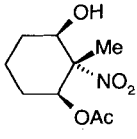
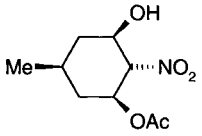
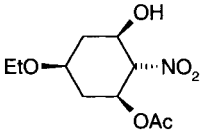
 44% ee, 54% yield	**1** [1]	 37% ee, 70% yield	**2** [2, 1]
 4% ee, 44% yield	**3** [1, 2]	 86% ee, 83% yield	**4** [1, 3, 4-6]
 8% ee, 40% yield	**5** [1]	 90% ee, 70% yield	**6** [7]
 13% ee, 75% yield	**7** [8]	 77% ee	**8** [8]
 4% ee, 31% yield	**9** [1, 9]	 55% ee, 60% yield 96% ee, 78% yield, *t*BuOH	**10** [1, 9]
 13% ee, 75% yield	**11** [10, 11]	 ≥95% ee, 80% yield	**12** [10]

Table 11.1-3. (cont.).



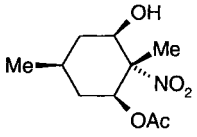
13 [10]

≥95 % ee, 70 % yield



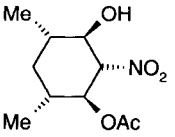
14 [10]

≥95 % ee, 60 % yield



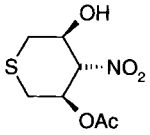
15 [10]

$[\alpha]_D$ + 9.8°, 68 % yield



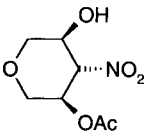
16 [10]

$[\alpha]_D$ - 1.3°, 60 % yield



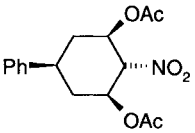
17 [10]

≥95 % ee, 60 % yield



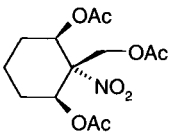
18 [10]

$[\alpha]_D$ + 14.7°, 20 % yield



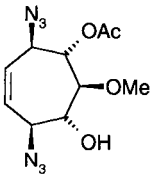
19 [10]

no hydrolysis



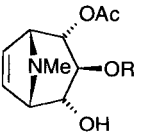
20 [10]

no hydrolysis



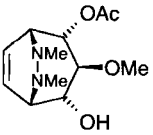
21 [2]

86 % ee, 88 % yield



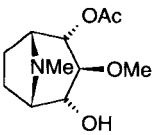
22 [12]

R = Me	99 % ee, 89 % yield
R = CH_2Ph	99 % ee, 100 % yield
R = CH_2OMe	99 % ee, 86 % yield



23 [12]

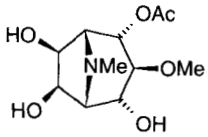
99 % ee, 89 % yield



24 [2]

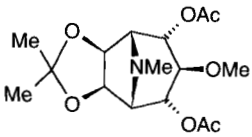
99 % ee, 100 % yield

Table 11.1-3. (cont.).



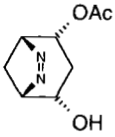
25 [12]

89% ee, 73% yield



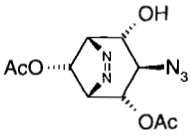
26 [12]

no hydrolysis



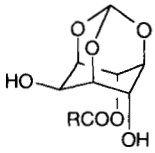
27 [12]

92% ee, 100% yield



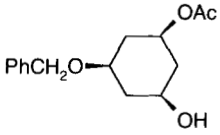
28 [12]

99% ee, 70% yield



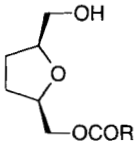
29 [13]

R = *n*Pr, ≥95% ee, 83% yield
R = Me, slow hydrolysis, racemate



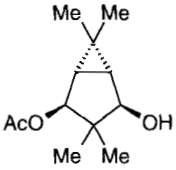
30 [14,15,16]

87% ee, 62% yield



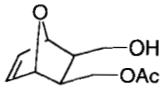
31 [17, 18, 19]

R = Me 96% ee, 86% yield
R = Et 42% ee, 54% yield
R = *i*Pr 94% ee, 62% yield
R = *t*Bu 55% ee, 72% yield



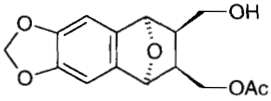
32 [20]

≥98% ee, 92% yield



33 [9]

57% ee
68% ee



34 [22]

33% ee, 52% yield
47% diacetate

1 K. Laumen, M. Schneider, *Tetrahedron Lett.* **1985**, *26*, 2073.
2 J. B. Jones, C. J. Francis, *Can. J. Chem.* **1984**, *62*, 2578.
3 Y. F. Wang, C. S. Chen, G. Girdaukas, C. J. Sih, *J. Am. Chem. Soc.* **1984**, *106*, 3695.
4 K. Laumen, M. Schneider, *Tetrahedron Lett.* **1984**, *25*, 5875.
5 Y. F. Wang, C. S. Chen, G. Girdaukas, C. J. Sih, F. S. Ciba, *J. Am. Chem. Soc.* **1985**, *111*, 128.
6 K. Laumen, E. H. Reimerdes, M. Schneider, *Tetrahedron Lett.* **1985**, *26*, 407.

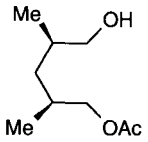
7 Y. F. Wang, C. J. Sih, *Tetrahedron Lett.* **1984**, *25*, 4999.
8 H. Suemune, T. Harabe, Z. F. Xie, K. Sakai, *Chem. Pharm. Bull.* **1988**, *36*, 4337.
9 G. Guanti, L. Banfi, E. Narisano, R. Riva, S. Thea, *Tetrahedron Lett.* **1986**, *27*, 4639.
10 M. Eberle, M. Egli, D. Seebach, *Helv. Chim. Acta* **1988**, *71*, 1.
11 D. Seeach, M. Eberle, *Chimia* **1986**, *40*, 315.
12 M. Falk-Heppner, M. Keller, H. Prinzbach, *Angew. Chem.* **1989**, *101*, 1281; *Angew. Chem., Int. Ed. Engl.* **1989**, *28*, 1253.
13 G. Baudin, B. J. Glänzer, K. S. Zwaminathan, H. Vasella, *Helv. Chim. Acta* **1988**, *71*, 1367.
14 M. Carda, J. Van der Eycken, M. Vandewalle, *Tetrahedron: Asymmetry* **1990**, *1*, 17.
15 H. Suemune, M. Takahashi, S. Maeda, Z. F. Xie, K. Sakai, *Tetrahedron: Asymmetry* **1990**, *1*, 425.
16 L. Dumortier, M. Carda, J. Van der Eycken, G. Snatzke, M. Vandewalle, *Tetrahedron: Asymmetry* **1991**, *2*, 789.
17 K. Naemura, N. Takahashi, H. Chikamatsu, *Chem. Lett.* **1988**, 1717.
18 H. Estermann, K. Prasad, M. J. Shapiro, O. Repic, M. J. Hardtmann, J. J. Bolsterli, M. D. Walkinshaw, *Tetrahedron Lett.* **1990**, *31*, 445.
19 K. Naemura, R. Fukada, N. Takahashi, M. Konishi, Y. Hirose, Y. Tobe, *Tetrahedron: Asymmetry* **1993**, *4*, 911.
20 A. Krief, D. Surleaux, N. Ropsan, *Tetrahedron: Asymmetry* **1993**, *4*, 289.
22 D. B. Berkowitz, J.-H. Maeng, *Tetrahedron: Asymmetry* **1996**, *7*, 1577.

formation of the corresponding chiral succinates (**94** and **95**) and glutarates (**96**) (Table 11.1-2). Exceptions are methyl 3-hydroxy-2,4-dimethyl glutarate (**97**) and methyl 1,2-dimethoxy succinate (**101**), which can be obtained with *ee* values of 98% and of 90%, respectively. Most interesting examples are the citric acid derivatives **87** and **102**. While the pig liver esterase-catalyzed hydrolysis of triethyl citrate proceeded with high enantioselectivity but low regioselectivity, that of the diester derivative of **102** occurred with both high regio- and enantioselectivity.

Acyclic monoesters of Table 11.1-2, which can be obtained with other hydrolases as such or of opposite configuration, are contained in Tables 11.1-7 and 11.1-12.

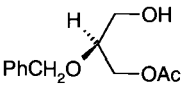
meso-Configured mono- and bicyclic diacetates, bearing primary or secondary acetoxy groups, are frequently hydrolyzed by pig liver esterase under standard

Table 11.1-4. Pig liver esterase-catalyzed enantiotopos-differentiating hydrolysis of prochiral acyclic diol diacetates in aqueous solution.



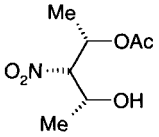
1 [1]

80% ee, 36% yield
95% ee, 15% yield



2 [2]

29% ee, 43% yield
39% ee, 54% yield



3 [3]

≥90% ee, 50% yield

1 Y. F. Wang, C. S. Chen, G. Girdaukas, C. J. Sih, *J. Am. Chem. Soc.* **1984**, *106*, 3695.
2 H. Suemune, Y. Mizuhara, H. Akita, K. Sakai, *Chem. Pharm. Bull.* **1986**, *34*, 3440.
3 D. Seebach, M. Eberle, *Chimia* **1986**, *40*, 315.

conditions at pH 7.0 to give the corresponding monoacetates (**1–34**) in high yields and with high enantioselectivities (Table 11.1-3). The examples listed in Table 11.1-3 demonstrate once again the low substrate specificity of pig liver esterase. At the same time the series of nitro substituted cyclohexanoid mono and diacetates **11–20** reveals that seemingly small changes in the structure of the substrate can suppress hydrolysis. Strategies to improve the enantioselectivity of pig liver esterase-catalyzed hydrolysis of a dialkyl dicarboxylate are as follows: a synthetically tolerable and meaningful substrate modification in the dicarboxylic acid part, a modification of the alcohol part of the substrate or the addition of an organic cosolvent. These strategies can also be applied in the case of acylated prochiral diols. While the polycyclic monoester **29**, which bears a butyryl group, is obtained with an *ee* value of ≥ 95 %, the derivative **29**, which carries an acetyl group, is formed as racemate. Enantioselectivity in the pig liver esterase-catalyzed formation of the cyclohexenoid monoacetate **10** can be dramatically improved if the hydrolysis of the corresponding diacetate is carried out in the presence of *tert*-butanol. The heterocyclic monoesters **31** demonstrate how, in the case of an diacylated diol, the achiral carboxylic acid part of the substrate influences the enantioselectivity of the hydrolysis. A series of highly functionalized cycloheptane derivatives (**21–26**) have been obtained through a pig liver esterase-catalyzed hydrolysis of the corresponding diacetates, with the same enantiotopic group recognition in all cases.

Cyclic monoacetates of Table 11.1-3, which can be obtained with other hydrolases as such or of opposite configuration, are contained in Tables 11.1-9, 11.1-11 and 11.1-18.

Only a very few acyclic prochiral acylated diols have been subjected with moderate success to pig liver esterase-catalyzed hydrolysis with formation of the corresponding chiral monoacetates (**1–3**) (Table 11.1-4). For this kind of compounds, lipases are the hydrolases of choice.

Acyclic monoacetates of Table 11.1-4, which can be obtained with other hydrolases as such or of opposite configuration, are contained in Tables 11.1-10 and 11.1-17.

Enantiomer-differentiating hydrolysis with pig liver esterase has, as with other hydrolases, become an important method for resolution (Table 11.1-5). Kinetic resolution of oxirane mono- and dicarboxylic acid esters with pig liver esterase proceeds effeciently with good selectivities, as demonstrated in the cases **14** and **15**. Resolution is of course not restricted to enantiomers with central chirality. Axial and planar chiral racemic ester have been resolved with moderate to good results with pig liver esterase (**33–36**).

Resolution of esters, the ester group of which is attached to a carbon atom bearing three other substituents, even when contained in a bi- or tricyclic ring system (**67**), represents no problem (Table 11.1-5). It seems interesting to note that these esters, which might be otherwise difficult to hydrolyze because of steric hindrance, are hydrolyzed readily via enzyme catalysis.

The cyclopentanoid esters **65** and **66** nicely illustrate how a seemingly remote functional group can significantly influence the enantioselectivity. Pig liver esterases allow for the kinetic resolution of α-hydroxy acids (**3–7**) and α-amino acids (**8–13**) which have a quaternary Cα-atom.

Table 11.1-5. Pig liver esterase-catalyzed enantiomer-differentiating hydrolysis of racemic carboxylic acid esters and lactones in aqueous solution (HLE horse liver esterase).

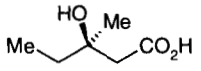
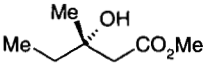
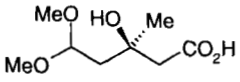
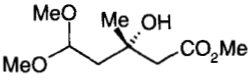
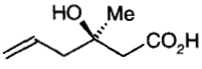
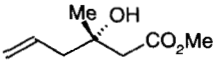
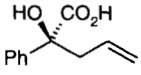
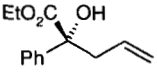
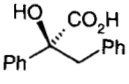
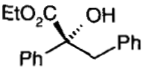
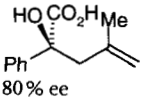
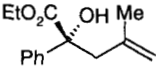
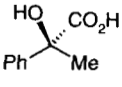
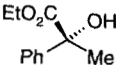
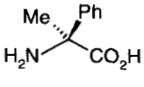
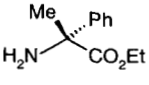
 low ee 88% conversion	**1a** [1]	 ≥98% ee, 12% yield	**1b** [1]
 low ee 67% conversion	**2a** [1]	 94% ee, 26% yield	**2b** [1]
 low ee	**3a** [1]	 94% ee, 11% yield	**3b** [1]
 75% ee 50% conversion	**4a** [2, 3, 4]	 64% ee 50% conversion	**4b** [2, 3, 4]
 38% ee	**5a** [2, 3]	 40% ee	**5b** [2, 3]
 80% ee	**6a** [2, 3]	 86% ee	**6b** [2, 3]
 51% ee	**7a** [2]	 40% ee	**7b** [2]
 31% ee, 6% yield	**8a** [5]	 5% ee, 67% yield	**8b** [5]

Table 11.1-5. (cont.).

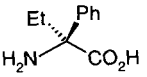
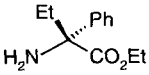
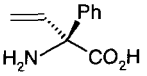
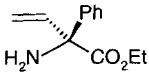
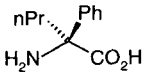
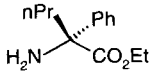
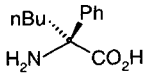
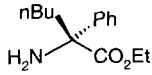
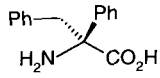
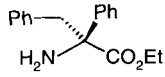
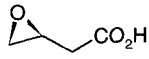
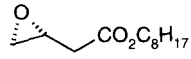
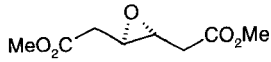
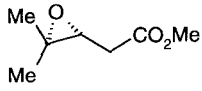
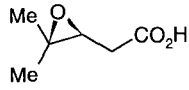
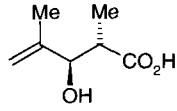
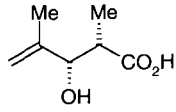
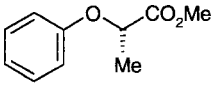
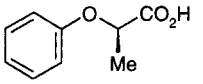
 13% ee, 40% yield	**9a** [5]	 25% ee, 28% yield	**9b** [5]
 72% ee, 57% yield	**10a** [5]	 95% ee, 41% yield	**10b** [5]
 17% ee, 23% yield	**11a** [5]	 20% ee, 50% yield	**11b** [5]
 93% ee, 41% yield	**12a** [5]	 97% ee, 31% yield	**12b** [5]
 54% ee, 39% yield	**13a** [5]	 61% ee, 10% yield	**13b** [5]
 74% ee, 30% yield 50% conversion	**14a** [6]	 82% ee, 40% yield	**14b** [6]
97% ee, 40% yield 50% conversion	**15a** [7]	 ≥95% ee, 40% yield	**15b** [7]
 E = 17	**16a** [8]	 E = 17	**16b** [8]
 64% ee 50% conversion	**17a** [9]	 63% ee	**17b** [9]

Table 11.1-5. (cont.).



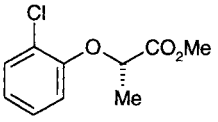
18a [10]

10% ee



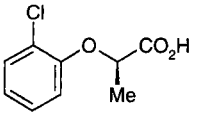
18b [10]

7% ee



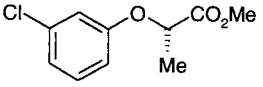
19a [10]

5% ee



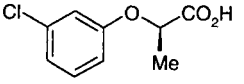
19b [10]

5% ee



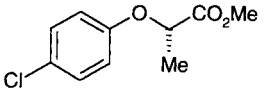
20a [10]

5% ee



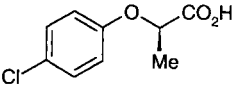
20b [10]

6% ee



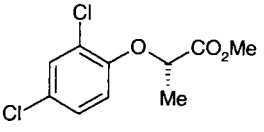
21a [10]

26% ee



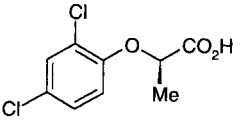
21b [10]

22% ee



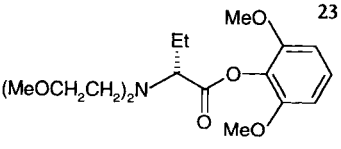
22a [10]

11% ee



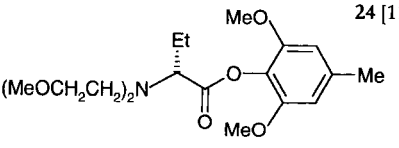
22b [10]

10% ee



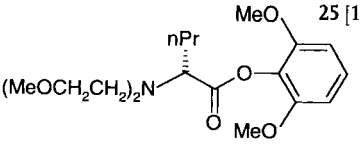
23 [11]

≥99% ee, 25–30% yield



24 [11]

≥99% ee, 25–30% yield



25 [11]

≥99% ee, 25–30% yield

Table 11.1-5. (cont.).

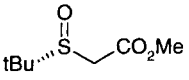
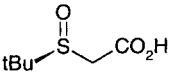
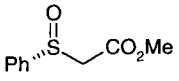
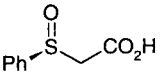
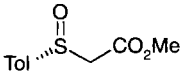
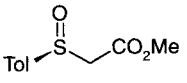
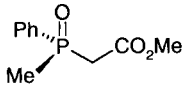
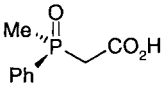
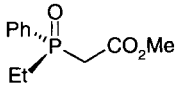
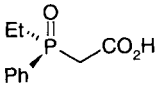
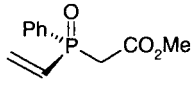
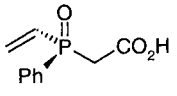
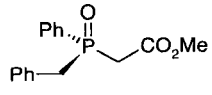
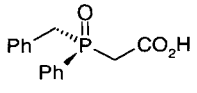
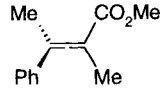
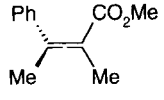
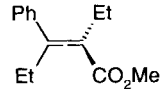
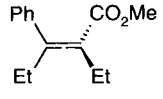
 48% ee, 53% yield	**26a** [12]	 38% ee, 38% yield	**26b** [12]
 21% ee, 52% yield	**27a** [12]	 34% ee, 40% yield	**27b** [12]
 80% ee, 32% yield	**28a** [12]	 46% ee, 58% yield	**28b** [12]
 73% ee, 50% yield	**29a** [13]	 82% ee, 42% yield	**29b** [13]
 96% ee, 45% yield	**30a** [13]	 81% ee, 41% yield	**30b** [13]
 ≥99% ee, 40% yield	**31a** [13]	 18% yield	**31b** [13]
 80% ee, 46% yield	**32a** [13]	 79% ee, 43% yield	**32b** [13]
 90% ee, 33% yield	**33a** [14]	 61% ee, 50% yield	**33b** [14]
 96% ee, 82% yield	**34a** [15]	 83% ee, 80% yield	**34b** [15]

Table 11.1-5. (cont.).

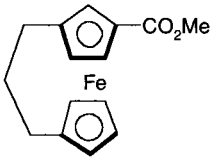
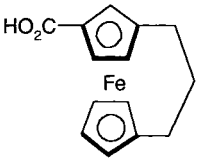
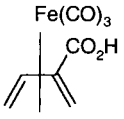
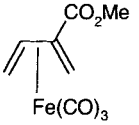
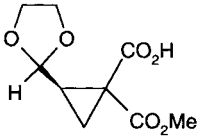
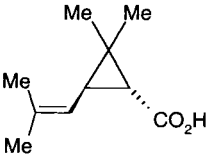
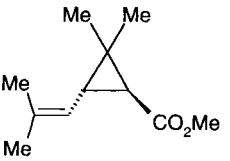
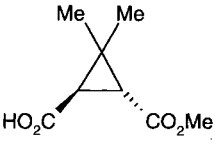
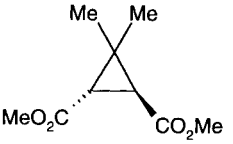
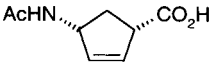
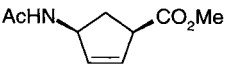
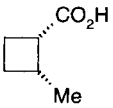
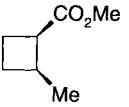
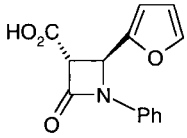
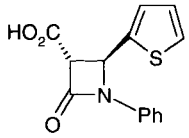
 70% ee	**35a** [16]	 75% ee	**35b** [16]
 85% ee 40% conversion	**36a** [14]	 85% ee 60% conversion	**36b** [14]
 86% ee 25% conversion	**37** [18]		
 36% ee	**38a** [19]	 40% ee	**38b** [19]
 60% ee	**39a** [19]	 50% ee, 50% yield	**39b** [19]
 97% ee, 47% yield	**40a** [20, 21, 22]	 87% ee, 43% yield	**40b** [20, 21, 22]
 ≥97% ee	**41a** [23]	 ≥97% ee	**41b** [23]

Table 11.1-5. (cont.).



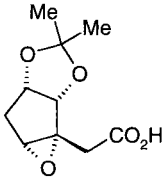
42 [24]

50% ee, 47% yield



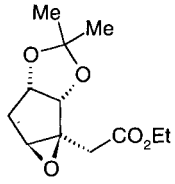
43 [24]

50% ee, 50% yield



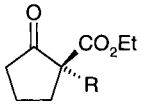
44a [25]

72% ee, 53% yield



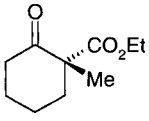
44b [25]

96% ee, 42% yield



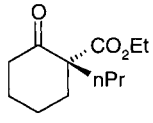
[26]

45 R = Me	0% ee
46 R = Et	93% ee, 66% yield
47 R = n-C_5H_{11}	97% ee, 50% yield
48 R = $(CH_2)_4CH{=}CH_2$	99% ee, 60% yield
49 R = n-C_9H_{19}	81% ee, 62% yield



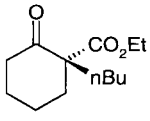
50 [27]

≥99% ee, 88% yield



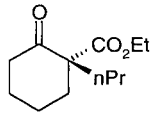
51 [27]

70% ee, 10% yield



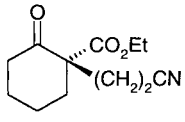
52 [27]

≥99% ee, 40% yield



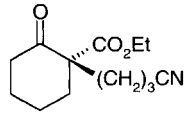
53 [27]

≥99% ee, 58% yield



54 [27]

≥99% ee, 70% yield



55 [27]

≥99% ee, 60% yield

Table 11.1-5. (cont.).

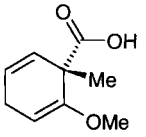
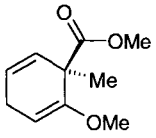
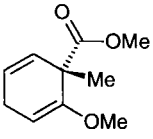
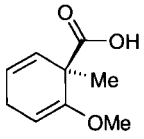
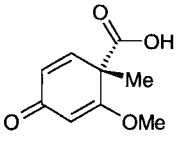
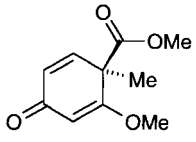
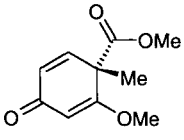
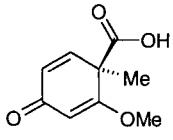
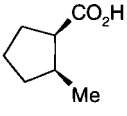
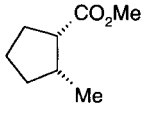
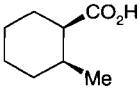
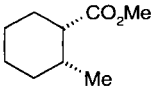
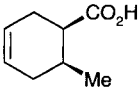
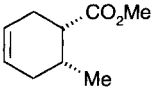
 32%	**56a** [28]	 93% ee, 36% yield	**56b** [28]
 96% ee, 42% yield, HLE	**57a** [28]	 34%, HLE	**57b** [28]
 30%	**58a** [28]	 94% ee, 36% yield	**58b** [28]
 92% ee, 38% yield, HLE	**59a** [28]	 34% yield, E = 65, HLE	**59b** [28]
 22% ee	**60a** [23]	 17% ee	**60b** [23]
 ≥97% ee	**61a** [23]	 ≥97% ee	**61b** [23]
 ≥97% ee	**62a** [23]	 ≥97% ee	**62b** [23]

Table 11.1-5. (cont.).

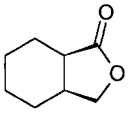
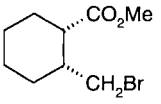
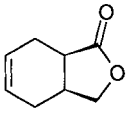
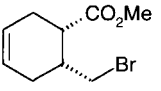
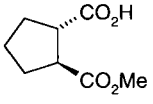
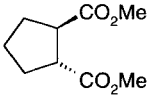
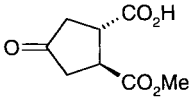
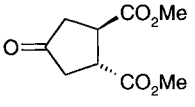
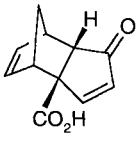
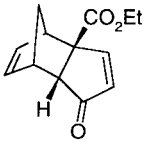
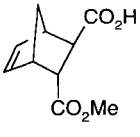
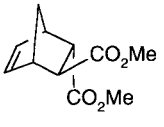
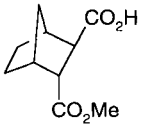
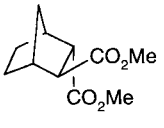
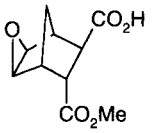
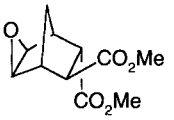
	**63a** [23]		**63b** [23]
≥97 % ee		≥97 % ee	
	**64a** [23]		**64b** [23]
≥97 % ee		≥97 % ee	
	**65a** [29]		**65b** [29]
59 % ee, 43 % yield		49 % ee, 54 % yield	
	**66a** [30]		**66b** [30]
95 % ee, 34 % yield 50 % conversion		95 % ee, 45 % yield	
	**67a** [31]		**67b** [31]
73 % ee, 45 % yield		≥99 % ee, 40 % yield	
	**68a** [32, 33, 34]		**68b** [32, 33, 34]
73 % ee, 45 % yield 64 % conversion 53 % ee, 55 % yield		70 % ee, 45 % yield 32 % ee, 95 % yield	
	**69a** [32, 33]		**69b** [32, 33]
82 % ee, 45 % yield		90 % ee, 40 % yield	

Table 11.1-5. (cont.).



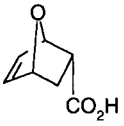
70a [32, 33]

82% ee, 45% yield



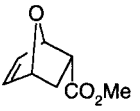
70b [32,33]

95% ee, 40% yield



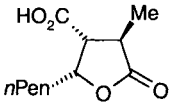
71a [35]

36% ee, 40% yield



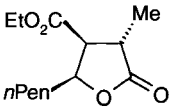
71b [35b]

57% ee, 48% yield



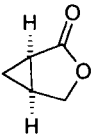
72a [36]

47% ee, 35% yield



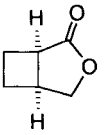
72b [36]

96% ee, 35% yield



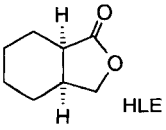
73 [37]

98% e, 41% yield, HLE



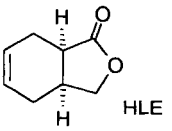
74 [37]

80% ee, 42% yield, HLE



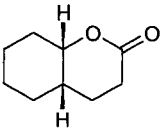
75 [37]

47% ee, 40% yield, HLE



76 [37]

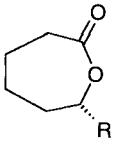
95% ee, 34% yield, HLE



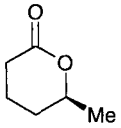
77 [37]

60% ee, HLE

Table 11.1-5. (cont.).

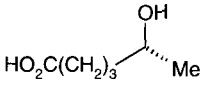


		PLE		HLE		
78	R = Et	98% ee	R	22	R	[38]
79	R = Pr	62% ee	R	4	R	[38]
80	R = *n*Bu	88% ee	R	38	S	[38]
81	R = *n*Pen	77% ee	R	53	S	[38, 39]
82	R = *n*Hex	33% ee	R	90	S	[38]
83	R = *n*Hep	60% ee	R	60	S	[38]
84	R = *n*Oct	65% ee	R	63	S	[38]



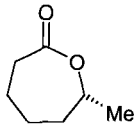
70% ee, 97% yield, PLE
95% ee, 90% yield, HLE

85a [40]



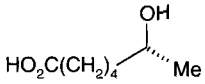
46% ee, 79% yield, PLE
64% ee, 83% yield, HLE

85b [40]



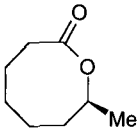
83% ee, 86% yield, PLE
76% ee, 80% yield, HLE

86a [40, 38]



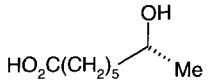
44% ee, 78% yield, PLE
47% ee, 84% yield, HLE

86b [40, 38]



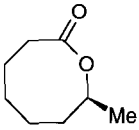
≥95% ee, 70% yield, PLE
≥95% ee, 74% yield, HLE

87a [40]



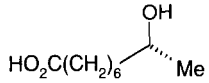
84% ee, 65% yield, PLE
42% ee, 71% yield, HLE

87b [40]



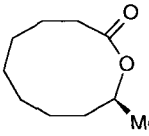
≥95% ee, 78% yield, PLE
≥95% ee, 94% yield, HLE

88a [40]



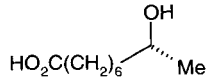
≥95% ee, 80% yield, PLE
≥95% ee, 84% yield, HLE

88b [40]



≥99% ee, 88% yield, HLE

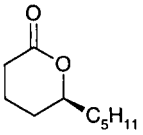
89a [40]



≥99% ee, 86% yield, HLE

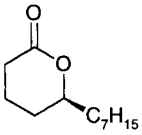
89b [40]

Table 11.1-5. (cont.).



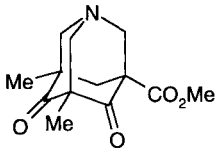
90 [37]

78% ee, HLE
18% ee, PLE



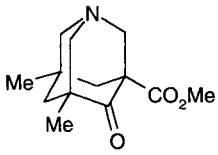
91 [37]

92% ee, HLE



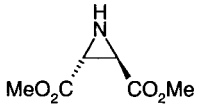
92a [41]

54% ee, 25% yield



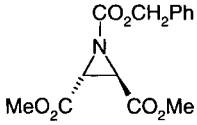
92b [41]

82% ee, 32% yield



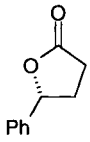
93a [42]

27% ee



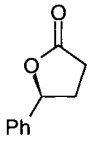
93b [42]

28% ee



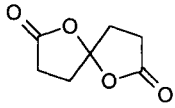
94a [43]

>94% ee, 90% yield



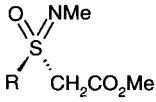
94b [43]

after lactonization
94% ee, 80% yield



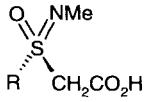
95 [43]

$[\alpha]_D^{20}$ + 31.9
≥90% ee



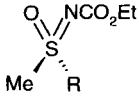
96a [44]

R = Ph, 12% ee, 25% yield
R = Tol, ≥90% ee, 12% yield



96b [44]

R = Ph, 10% ee, 45% yield
R = Tol, 13% ee, 70% yield



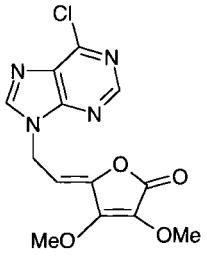
97a [44]

R = Ph, 14% ee, 50% yield

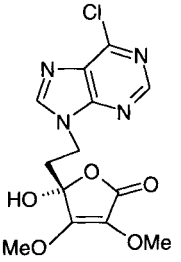
97b [44]

R = Ph, 41% ee, 12% yield

Table 11.1-5. (cont.).

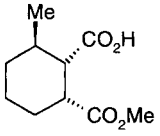


98a [45]

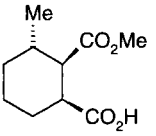


98b [45]

95 % yield
$[\alpha]^{D}_{25}$ -15.4 (1.02, MeOH)



99a [46]



99b [46]

≥95 % ee

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28 C. Tanyeli, B. Sezen, A. S. Demir, R. B. Alves, A. Arseniyadis, *Tetrahedron: Asymmetry* **1999**, *10*, 1129.
29 Y. Morimoto, Y. Terao, K. Achiwa, *Chem. Pharm. Bull.* **1987**, *35*, 2266.
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Phosphorus- and to a lesser extent sulfur-containing racemic esters could be resolved with pig liver esterase (**29–32** and **26–28**) as well. Particularly interesting examples are the β-keto esters (**45–54**), which were frequently obtained with high enantioselectivities. The enantiomer, which was hydrolyzed preferentially, suffered a decarboxylation with formation of the corresponding ketone. Interesting examples, showing the strategy in the application of hydrolases in kinetic resolution, are the cyclohexadiene-carboxylates **56–59**. The use of both pig liver esterase and horse liver esterase (HLE) allows for the attainment of both enantiomers of both the acid and the ester. In the resolution of the racemic esters **60b–62b**, pig liver esterase shows in regard to the configuration of the C-atom to which the ester group is bound the same preference as in the case of the corresponding *meso*-diesters (Table 11.1-1). This also holds true for the resolution of the racemates of esters **63b** and **64b**, which carry a bromomethyl group instead of the methyl group. Here, the enantiomer, which carries the carboxy group, suffers lactonization with formation of lactones **63a** and **64a**. A most remarkable example of a pig liver esterase-catalyzed reaction is the apparent enantioselective hydration of alkene **98a** with formation of the hydroxy lactone **98b**. The mechanism of this reaction is not known. It seems interesting to note in this context that reaction of **98a** with aqueous ammonia affords racemic **98b**. Finally, the pig liver esterase-catalyzed hydrolysis of the racemic diester derivatives of **99a** and **99b** shows the remarkable feature that the two enantiomers are hydrolyzed with different regioselectivities.

A large number of mono- and bicyclic lactones (**73–91**) have been obtained by using pig liver esterase in combination with horse liver esterase for the enantiomer-differentiating hydrolysis of the corresponding racemic lactones. Interestingly, in the series of methyl-substituted lactones (**85–89**), both enzymes show toward the seven-membered lactone (**86**) the opposite enantiomer selectivity as compared to the other lactones.

Acids or esters of Table 11.1-5, which can be obtained with other hydrolases as such or of opposite configuration, are contained in Table 11.1-13.

Kinetic resolution by pig liver esterase is not restricted to mono- and dicarboxylic acid ester derivatives. Acetates of racemic secondary alcohols are also excellent

Table 11.1-6. Pig liver esterase-catalyzed enantiomer-differentiating hydrolysis of esters of racemic alcohols in aqueous solution.

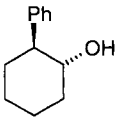
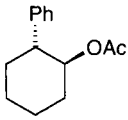
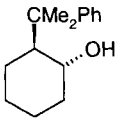
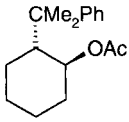
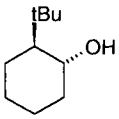
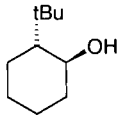
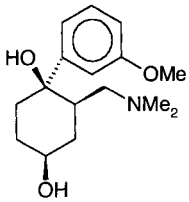
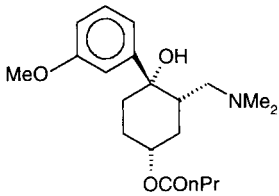
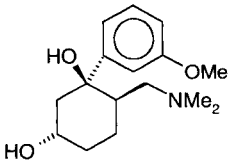
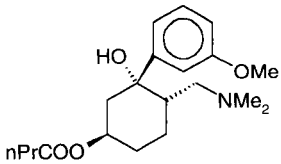
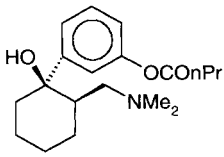
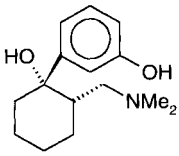
	**1a** [1, 2]		**1b** [1, 2]
98% ee, 40% yield		98% ee, 53% yield	
	**2a** [2]		**2b** [2]
67% ee, 36% yield		95% ee, 44% yield	
	**3a** [3]		**3b** [3]
≥99% ee after crystallization		≥99% ee after crystallization	
	**4a** [4]		**4b** [4]
93% ee, 96% yield		72% ee, 99% yield	
	**5a** [4]		**5b** [4]
94% ee, 77% yield		86% ee, 79% yield	
	**6a** [4]		**6b** [4]
76% ee		62% ee	

Table 11.1-6. (cont.).

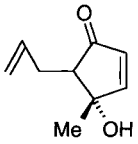
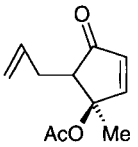
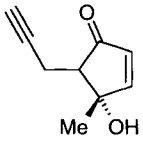
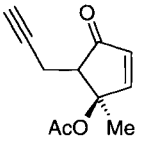
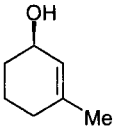
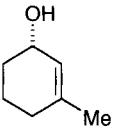
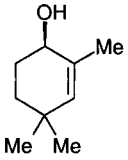
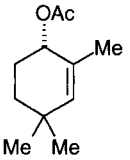
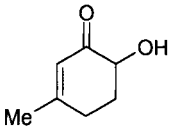
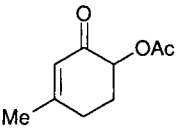
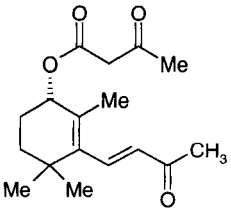
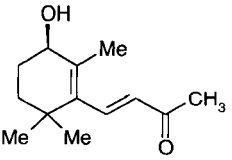
 98% ee	**7a** [5]	 –	**7b** [5]
 98% ee	**8a** [5]		**8b** [5]
 ≥95% ee, 12% yield	**9a** [6]	 ≥95% ee, 28% yield	**9b** [6]
 100% ee	**10a** [7]	 41% ee	**10b** [7]
 ≥95% ee, 43% yield 43% conversion	**11a** [8]	 ≥88% ee, 40% yield 43% conversion	**11b** [8]
 27% ee	**12a** [9]	 94% ee, 32% yield	**12b** [9]

Table 11.1-6. (cont.).

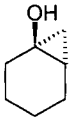
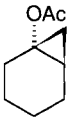
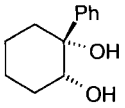
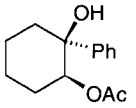
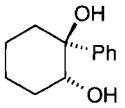
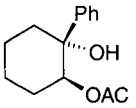
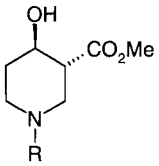
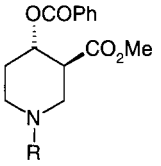
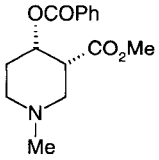
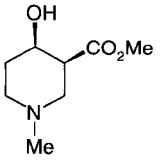
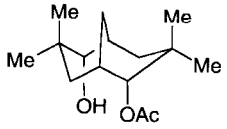
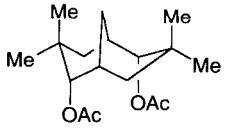
	**13a** [10]		**13b** [10]
64% ee		91% ee, E = 14	
	**14a** [11, 12]		**14b** [11, 12]
84% ee		85% ee	
	**15a** [11, 12]		**15b** [11, 12]
78% ee		82% ee	
	**16a** [13]		**16b** [13]
99% ee, 55% yield 40% ee, 38% yield		100% ee, 69% yield 95% ee, 40% yield	
	**17a** [13]		**17b** [13]
99% ee, 71% yield		97% ee, 45% yield	
	**18a** [14, 15]		**18b** [14, 15]
96% ee, 43% yield 50% conversion		86% ee, 46% yield	

Table 11.1-6. (cont.).

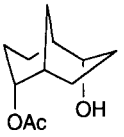
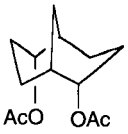
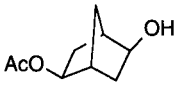
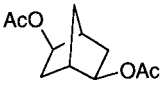
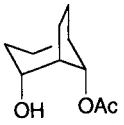
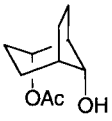
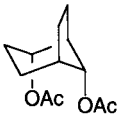
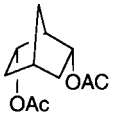
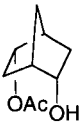
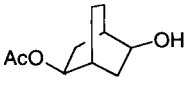
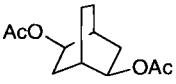
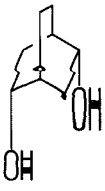
	**19a** [14, 15]		**19b** [14, 15]
31% ee, 43% yield 50% conversion		31% ee, 43% yield	
	**20a** [16]		**20b** [16]
10% ee		13% ee	
	**21a** [16]		**21b** [16]
87% ee		85% ee	
	**21c** [16]		
8% ee			
	**22a** [16]		**22b** [16]
15% ee		19% ee	
	**23a** [16]		**23b** [16]
21% ee		26% ee	
	**24a** [16]		**24b** [16]
36% ee		84% ee	

Table 11.1-6. (cont.).

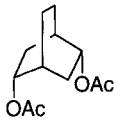
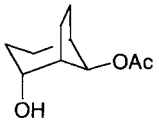
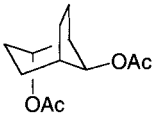
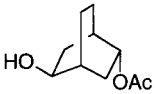
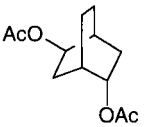
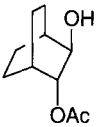
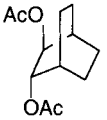
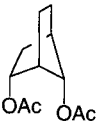
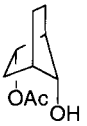
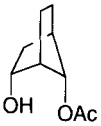
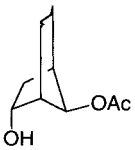
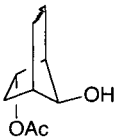
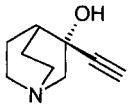
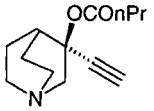
 66 % ee	**24c** [16]		
 87 % ee	**25a** [16]	 48 % ee	**25b** [16]
 17 % ee	**26a** [16]	 17 % ee	**26b** [16]
 82 % ee	**27a** [16]	 85 % ee	**27b** [16]
 81 % ee	**28a** [16]	 98 % ee	**28b** [16]
 73 % ee	**28c** [16, 17]		
 43 % ee	**29a** [16, 17]	 83 % ee	**29b** [16, 17]

Table 11.1-6. (cont.).



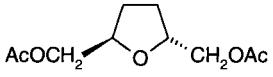
30a [18]

97 % ee, 36 % yield
35 % conversion



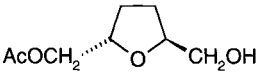
30b [18]

99 % ee, 40 % yield
56 % conversion



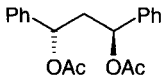
31a [19]

58 % ee, 48 % yield



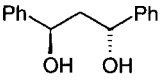
31b [19]

58 % ee, 47 % yield



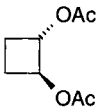
32a [20]

84 % ee, 43 % yield
5 % monoacetate



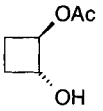
32b [20]

92 % ee, 40 % yield



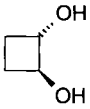
33a [20]

≥95 % ee, 41 % yield
30 % conversion



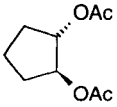
33b [20]

≥95 % ee, 49 % yield



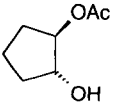
33c [20]

≥95 % ee, 10 % yield



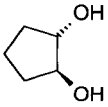
34a [20]

54 % ee, 54 % yield
25 % conversion



34b [20]

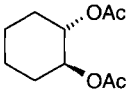
50 % ee, 43 % yield
25 % conversion



34c [20]

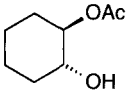
47 % ee, 53 % yield
74 % conversion

Table 11.1-6. (cont.).



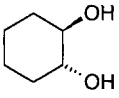
35a [20, 21]

≥95% ee, 33% yield
25% conversion



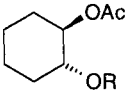
35b [20, 21]

0% ee, 26% yield

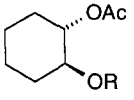


35c [20, 21]

≥95% ee, 41% yield

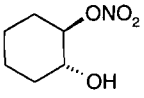


[22,23]



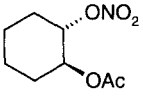
[22,23]

36a	R = Ph	98% ee, 32% yield	**36b**	85% ee, 42% yield
37a	R = *p*-MeC_6H_4	≥99% ee, 30% yield	**37b**	70% ee, 41% yield
38a	R = *p*-*t*BuC_6H_4	≥99% ee, 33% yield	**38b**	90% ee, 36% yield
39a	R = *p*-PhC_6H_4	≥99% ee, 34% yield	**39b**	88% ee, 37% yield
40a	R = *o*-$MeOC_6H_4$	92% ee, 47% yield	**40b**	77% ee, 45% yield
41a	R = 2,4-$Me_2C_6H_4$	90% ee yield	**41b**	60% ee



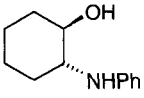
42a [24]

≥99% ee, 35% yield, 3d, PLAP
66% ee, 55% yield, 6d, PLAP



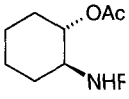
42b [24]

71% ee, 52% yield, 3d, PLAP
≥99% ee, 36% yield, 6d, PLAP



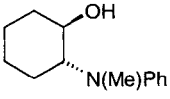
43a [25]

≥99% ee, 48%



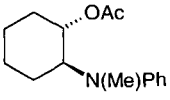
43b [25]

91% ee, 50%, E >637



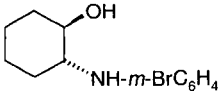
44a [25]

≥99% ee, 48% yield



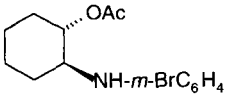
44b [25]

80% ee, 51% yield, E >477



45a [25]

≥99% ee, 38% yield



45b [25]

57% ee, 61% yield, E >425

Table 11.1-6. (cont.).

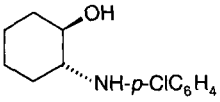
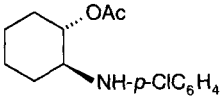
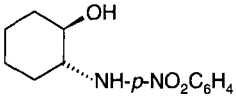
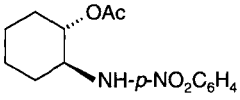
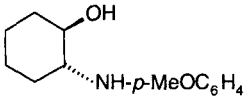
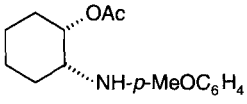
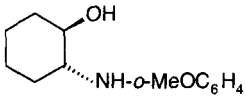
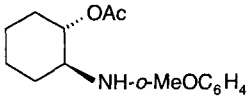
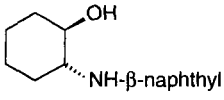
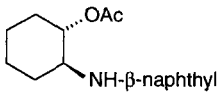
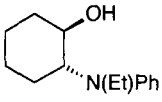
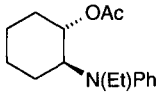
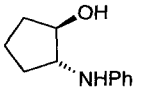
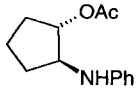
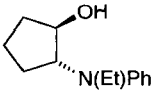
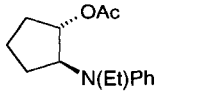
	**46a** [25]		**46b** [25]
≥99% ee, 46% yield		96% ee, 49% yield, E >989	
	**47a** [25]		**47b** [25]
94% ee, 32% yield		57% ee, 63% yield, E >57	
	**48a** [25]		**48b** [25]
79% ee, 52% yield		97% ee, 46% yield, E >39	
	**49a** [25]		**49b** [25]
73% ee, 37% yield		42% ee, 56% yield	
	**50a** [25]		**50b** [25]
≥99% ee, 34% yield		52% ee, 62% yield, E >335	
	**51a** [25]		**51b** [25]
95% ee, 32% yield		51% ee, 51% yield, E = 65	
	**52a** [25]		**52b** [25]
65% ee, 60% yield		87% ee, 40% yield, E = 13	
	**53a** [25]		**53b** [25]
52% ee, 50% yield		68% ee, 58% yield, E = 6	

Table 11.1-6. (cont.).

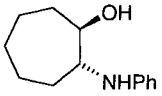
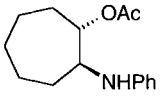
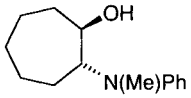
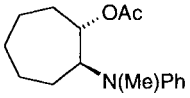
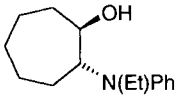
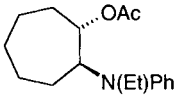
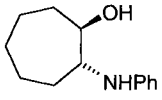
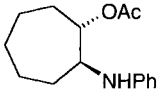
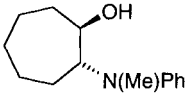
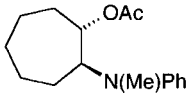
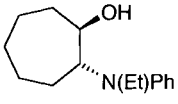
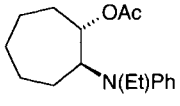
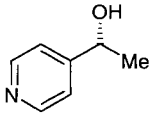
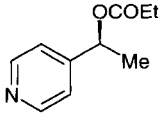
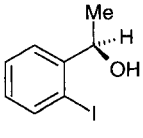
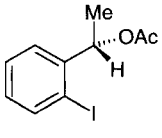
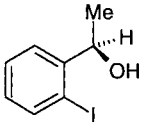
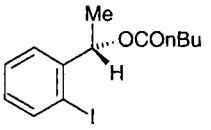
	**54a** [25]		**54b** [25]
92% ee, 45% yield		99% ee, 52% yield, E = 126	
	**55a** [25]		**55b** [25]
94% ee, 33% yield		68% ee, 64% yield, E = 67	
	**56a** [25]		**56b** [25]
≥99% ee, 38% yield		70% ee, 55% yield, E >419	
	**57a** [25]		**57b** [25]
89% ee, 34% yield		57% ee, 52% yield, E = 22	
	**58a** [25]		**58b** [25]
≥99% ee, 25% yield		39% ee, 50% yield, E >292	
	**59a** [25]		**59b** [25]
≥99% ee, 25% yield		37% ee, 73% yield, E >295	
	**60a** [26]		**60b** [26]
88% ee, 44% yield		98% ee, 43% yield	
	**61a** [27]		**61b** [27]
21% ee, 60% yield		32% ee, 36% yield, E = 1,7	

Table 11.1-6. (cont.).



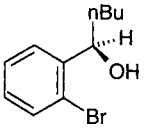
86 % ee, 48 % yield

62a [27]



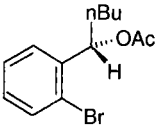
83 % ee, 49 % yield, E = 40

62b [27]



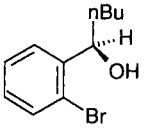
50 % ee, 38 % yield

63a [27]



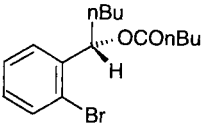
30 % ee, 54 % yield, E = 7,6

63b [27]



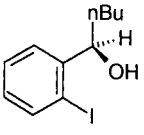
40 % ee, 40 % yield

64a [27]



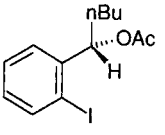
30 % ee, 44 % yield, E = 3,8

64b [27]



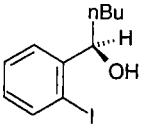
39 % ee, 49 % yield

65a [27]



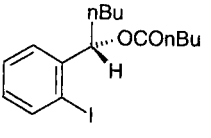
33 % ee, 49 % yield, E = 3,8

65b [27]



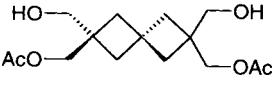
60 % ee, 45 % yield

66a [27]



50 % ee, 48 % yield, E = 8,5

66b [27]

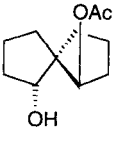


56 % ee

67 [28]

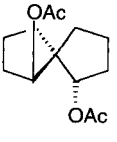
64 % ee

68 [28]



24 % ee

69a [28]



31 % ee

69b [28]

Table 11.1-6. (cont.).

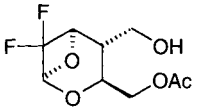
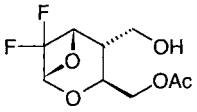
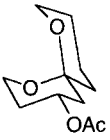
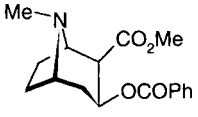
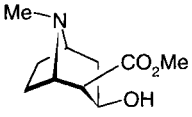
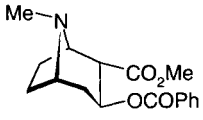
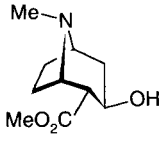
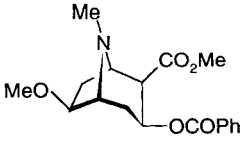
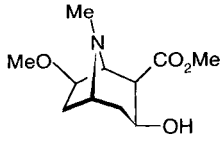
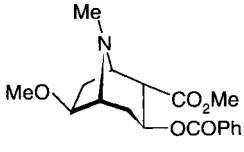
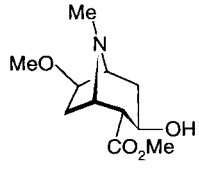
	**70a** [29c]		**70b** [29c]
100% ee, 24% yield		100% ee, 24% yield	
	71a [30]		**71b** [30]
≥99% ee after hydrolysis, acylation and hydrolysis -5 to -10 °C, MeOH		≥99% twofold hydrolysis	
	**72a** [31, 32]		**72b** [31, 32]
82% ee, 45% yield		71% ee, 35% yield	
	**73a** [31, 32]		**73b** [31, 32]
≥99% ee, 91% yield		95% ee, 85% yield	
	**74a** [31, 32]		**74b** [31, 32]
82% ee, 35% yield		95% ee, 3% yield	
	**75a** [31, 32]		**75b** [31, 32]
99% ee, 60% yield		99% ee, 30% yield	

Table 11.1-6. (cont.).

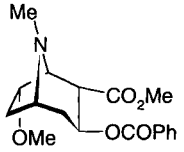
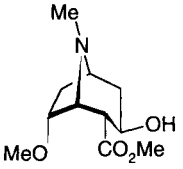
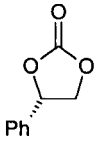
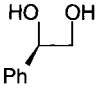
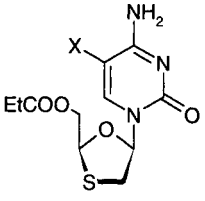
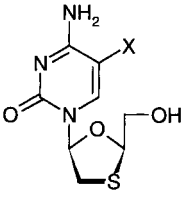
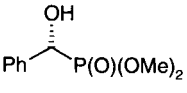
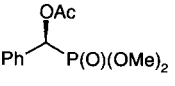
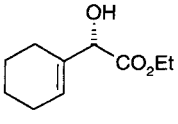
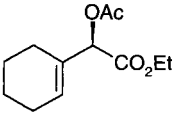
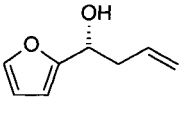
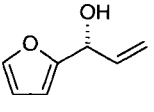
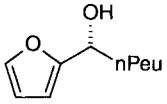
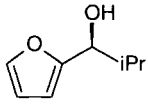
 97 % ee, 55 % yield	**76a** [31, 32]	 95 % ee, 26 % yield	**76b** [31, 32]
 78 % ee	**77a** [33]	 97 % ee	**77b** [33]
 R = H ≥99 % ee R = F 93 % ee 50 % conversion	**78a** [34]	 - -	**78b** [34]
 7 % ee, 32 % yield 46 % conversion	**79a** [35]	 37 % ee, 3 % yield 46 % conversion	**79b** [35]
 90 % ee, 81 % yield	**80a** [36]	 54 % ee	**80b** [36]
 93 % ee 47 % conversion	**81** [37]	 98 % ee 45 % conversion	**82** [37]

Table 11.1-6. (cont.).



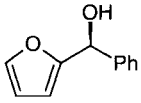
83 [37]

22% ee
42% conversion



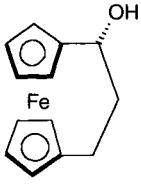
84 [37]

96% ee
45% conversion



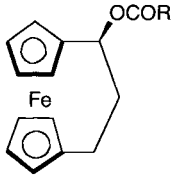
85 [37]

22% ee
48% conversion



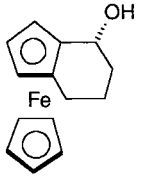
86a [38]

R = Me 36% ee, 55% yield
R = *n*Pr 1% ee, 33% yield



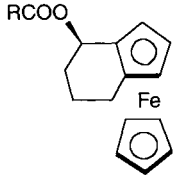
86b [38]

78% ee, 26% yield, E = 5
2% ee, 56% yield, E = 1



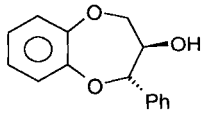
87a [38]

R = Me 91% ee, 43% yield
R = *n*Pr



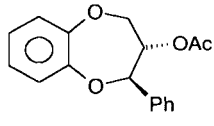
87b [38]

94% ee, 43% yield, E = 75



88a [39]

75% ee, 50% yield, PLAP



88b [39]

87% ee, 50% yield, PLAP

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9 J. Y. Lallemand, M. Leclaire, R. Levet, G. Aranda, *Tetrahedron: Asymmetry* **1993**, *4*, 1775.
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14 K. Naemura, T. Matsumura, M. Komatsu, Y. Hirose, H. Chikamatsu, *Bull. Chem. Soc. Jpn.* **1989**, *62*, 3523.
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16 K. Naemura, N. Takahashi, S. Tanaka, H. Ida, *J. Chem. Soc., Perkin Trans. 1* **1992**, 2337.
17 K. Naemura, N. Takahashi, S. Tanaka, M. Ueno, H. Chikamatsu, *Bull. Chem. Soc. Jpn.* **1990**, *63*, 1010.
18 J. F. Coope, B. G. Main, *Tetrahedron: Asymmetry* **1995**, *6*, 1393.
19 K. Naemura, R. Fukuda, N. Takahashi, M. Konishi, Y. Hirose, Y. Tobe, *Tetrahedron: Asymmetry* **1993**, *4*, 911.
20 K. Yamamoto, H. Ando, H. Chikamatsu, *J. Chem. Soc., Chem. Commun.* **1987**, 334.
22 D. Basavaiah, P. R. Krishna, T. K. Bharathi, *Tetrahedron: Asymmetry* **1995**, *2*, 439.
21 G. Caron, R. J. Kazlauskas, *J. Org. Chem.* **1991**, *56*, 7251.
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26 P. Rasor, C. Rüchardt, *Chem. Ber.* **1989**, *122*, 1375.
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31 A. P. Kozikowski, D. Simoni, P. G. Baraldi, I. Lampronti, S. Manfredini, *Bioorg. Med. Chem. Lett.* **1996**, *6*, 441.
32 D. Simoni, M. Roberti, V. Andrisano, M. Manferdini, R. Rondanin, F. P. Invidiata, *Farmaco* **1999**, *54*, 275.
33 P. Barton, M. I. Page, *Tetrahedron* **1992**, *48*, 7731.
34 L. K. Hoong, L. E. Strange, D. C. Liotta, G. W. Koszalka, C. L. Burns, R. F. Schinazi, *J. Org. Chem.* **1992**, *57*, 5563.
35 Y. F. Li, F. Hammerschmidt, *Tetrahedron: Asymmetry* **1993**, *4*, 109.
36 P. S. Vankar, I. Bhattacharya, Y. D. Vankar, *Tetrahedron: Asymmetry* **1996**, *6*, 1683.
37 C. Tanyeli, A. S. Demir, A. H. Arkin, I. M. Akhmedov, *Enantiomer* **1997**, *2*, 433.
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substrates for pig liver esterase in terms of an efficient resolution. In particular, pig liver esterase is, as well as lipases, the hydrolase of choice for the kinetic resolution of secondary cyclic alcohols and in particular of cyclohexane derivatives. This is impressively demonstrated by the many examples contained in Table 11.1-6. Quite a number of enantioenriched β-amino alcohols (**43–59**) having different ring size have been obtained in this manner. Pig liver esterase seems to be especially well suited for the kinetic resolution of cyclic 1,2-diols (**14**, **15**, **33–42**). In the case of 1,2-diols, having C_2-symmetry, sequential kinetic resolution can be applied for *ee* enhancement. Resolutions of the racemates of **42b** and **88b** show the successful use of a crude extract of pig liver (PLAP). Further interesting examples are the resolution of cocaine derivatives (**72–76**) and of amino alcohols (**4–6**). In the case of the resolution of the racemate of **6a**, the remote butyroxy group attached to the aromatic ring is hydrolyzed. Within the series of racemic acetates which have been subjected to liver esterase-catalyzed hydrolysis (Table 11.1-6), the cyclohexanoid compounds **1–3** are particularly interesting since they are valuable chiral auxiliaries. Somewhat puzzling results were recorded in the case of cyclic 1,2-diacetates with homotopic acetoxy groups. Selectivity is lowest in the case of the five-membered compound, and, not

surprisingly, diol formation is in all cases significant. As observed in the case of cyclic *meso*-dicarboxylic acid diesters (Table 11.1-1), there is a reversal in the sense of asymmetric induction on going from the four-membered to the six-membered diacetates. Especially noteworthy is the observation that racemic tertiary acetates are also amenable to kinetic resolution with pig liver esterase (**7**, **8**, **13**, and **30**). Kinetic resolution of esters of acyclic alcohols with pig liver esterase has been studied only to a minor extent. However, some of the examples described proceeded highly selectively (**60**, **62**, **81**, **82**, and **84**).

Acylated alcohols and alcohols of Table 11.1-6, which can be obtained with other hydrolases as such or of opposite configuration, are contained in Tables 11.1-15, 11.1-16, 11.1-20 and 11.1-21.

11.1.1.1.2 α-Chymotrypsin

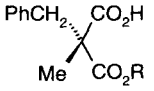
α-Chymotrypsin (CHT, E.C. 3.4.21.1) is one of the most thoroughly studied hydrolases[1, 4, 9, 12, 21, 23, 26, 28, 33, 34], and its crystal structure has been determined[83]. It is a serine protease with a pH optimum of 7.8, which acts *in vivo* as an endopeptidase and catalyzes with great specificity the hydrolysis of non-terminal amide bonds adjacent to phenylalanine, tyrosine or tryptophan. The enzyme has been widely used for the kinetic resolution of racemic amino acid esters. From the results of these studies and based on the crystal structure of the enzyme a useful active site model for α-chymotrypsin has been developed[1, 81]. Hydrolyses catalyzed by α-chymotrypsin are usually carried out in aqueous buffer solution in a pH range of 7-8. In the case of a low solubility of the substrate in water cosolvents such as methanol, ethanol, dimethylformamide or dimethylsulfoxide, up to 20% may be used. However, it should be noted that primary alcohols used as cosolvents may react with the acyl-enzyme intermediate with formation of the corresponding ester (Scheme 11.1-9). Diethyl ether, even in low concentrations, is a strong inhibitor of the enzyme. Immobilization of α-chymotrypsin by different methods has been described and the immobilized enzyme is commercially available. α-Chymotrypsin has been found to be active in organic solvents of low water content also[108].

A limited number of prochiral malonates and glutarates are hydrolyzed by α-chymotrypsin to the corresponding monoesters with synthetically useful enantioselectivities(**1–9**) (Table 11.1-7).

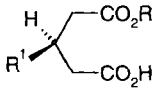
Examples of enantioselective hydrolysis of cyclic diesters by α-chymotrypsin are comparatively rare (**10–14**) (Table 11.1-7). Interestingly, the cyclopentanoid and the cyclohexenoid monoesters **11** and **12** have the opposite absolute configuration to those obtained by the pig liver esterase-catalyzed hydrolysis of the corresponding diesters (Table 11.1-1). The keto ester **14**, which is a valuable building block for the synthesis of prostacyclin analogs, has been obtained from the corresponding α,α'-keto diester via α-chymotrypsin-catalyzed hydrolysis followed by a decarboxylation of the keto acid.

Monoesters and monoacetates of Table 11.1-7, which can be obtained with other hydrolases as such or of opposite configuration, are contained in Tables 11.1-1, 11.1-2, 11.1-3, 11.1-9, 11.1-11 and 11.1-18.

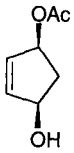
Table 11.1-7. α-Chymostrypsin-catalyzed enantiotopos-differentiating hydrolysis of prochiral cyclic dicarboxylic acid esters, acyclic dicarboxylic acid esters and cyclic diol diacetates and enantiomer-differentiating hydrolysis of racemic carboxylic acid esters in aqueous solution.



1 [1] R = CH_3 ≥98% ee, 90-98% yield, DMSO
2 [1] R = C_2H_5 ≥98% ee, 90-98% yield, DMSO

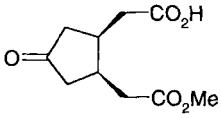


	R^1	R^2	ee (%)	yield (%)	Ref.
3	HO	CH_3	64	100	[2–5]
4	C_6H_5COO	CH_3	84	68	[4]
5	$C_6H_5CH_2$	CH_3	92	86	[4]
6	CH_3OCH_2	CH_3	93	100	[4]
7	CH_3COO	CH_3	90	38	[5]
3	HO	CH_3	55	78	[5]
8	CH_3COO	C_2H_5	95	84	[5]
9	CH_3CONH	C_2H_5	≥95	79	[6]



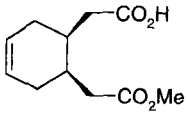
10 [7]

42% ee, 73% yield



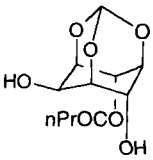
11 [8]

83% ee, 87% yield



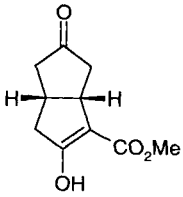
12 [8]

86% ee, 90% yield



13 [9]

40% ee, –



14 [10]

≥95% ee, 60% yield

Table 11.1-7. (cont.).

$R^2-CH(NHR^1)-CO_2R^3$ (±)

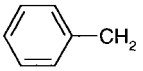
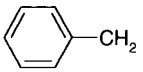
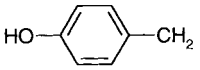
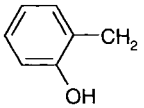
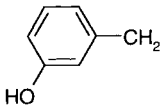
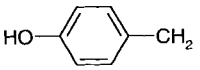
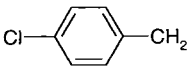
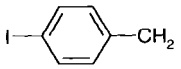
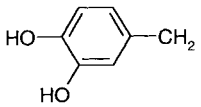
R^1	R^2	R^3	L-acid op (%), yield (%)	D-ester op (%), yield (%)	Ref.
15a, b $COCH_3$		CH_3	≥95, 88	≥95, 68	[11]
16a, b CHO		CH_3	≥95, 92	–	[12, 13]
17a, b $COCH_3$		CH_3	–	≥95, 69	[14]
18a, b H		C_2H_5	≥95, 75	≥95, 50	[15]
19a, b H		C_2H_5	≥95, 75	≥95, 78	[15]
20a, b H		C_2H_5	≥95, 80	≥95, 78	[15]
21a, b H		C_2H_5	≥95, 60	≥95, 40	[15]
22a, b H		C_2H_5	≥95, 60	≥95, 60	[15]
23a, b H		C_2H_5	≥95, 70	≥95, 60	[15]

Table 11.1-7. (cont.).

$R^2-CH(NHR^1)-CO_2R^3$ (±)

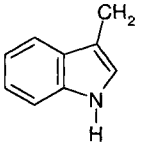
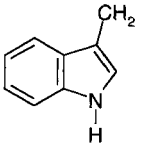
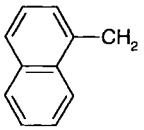
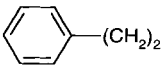
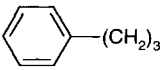
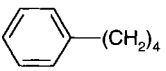
R^1	R^2	R^3	L-acid op (%), yield (%)	D-ester op (%), yield (%)	Ref.
24a, b H		CH_3	≥95, 87	≥95, 70	[16]
25a, b $COCH_3$		CH_3	97, 60	– 100	[17]
26a, b $COCH_3$	$CH_3(CH_2)_4$	CH_3	≥95, 40	≥95, 50	[18]
27a, b $COCH_3$		CH_3	high, 42	high, 45	[19]
28a, b $COCH_3$		CH_3	≥95, 53	≥95, 60	[20]
29a, b $COCH_3$		C_2H_5	84, 28	high, 93	[21]
30a, b $COCH_3$		CH_3	≥98, 61	86, 58	[20]
31a, b $COCH_3$		CH_3	≥90, 65	≥95, 86	[20]
32a, b $COCH_3$		CH_3	≥95, 65	≥95, 81	[20]

Table 11.1-7. (cont.).

$R^2-CH(NHR^1)-CO_2R^3$ (±)

R^1	R^2	R^3	L-acid op (%), yield (%)	D-ester op (%), yield (%)	Ref.
33a, b $COCH_3$	CH_3	C_2H_5	≥95, 91	≥95, 92	[21]
34a, b $COCH_3$	$C_2H_5OCOCH_2$	C_2H_5	≥95, 97	≥95, 83	[22]
35a, b $COCH_3$	$C_2H_5OCO(CH_2)_2$	C_2H_5	≥95, 74	≥95, 68	[23]
36a, b $COCH_3$	NO_2	CH_3		≥95,–	[24]

$R^2(R^3)C(R^1)-CO_2R^4$ (±)

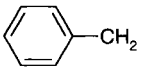
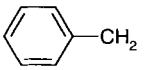
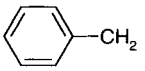
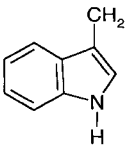
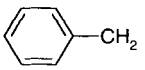
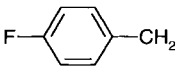
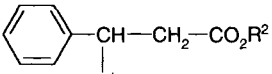
R^1	R^2	R^3	L-acid op (%), yield (%)	D-ester op (%), yield (%)	Ref.
37a, b $OCOCH_3$		H	low, 100	low, 40	[25]
38a, b CH_3		H	≥95, 83	≥95, 88	[26]
39a, b OH		CH_3	high, 100	75, 91	[27]
40a, b CH_3		NH_2	≥95, 72	≥95, 88	[28]
41a, b CH_3		NH_2	≥95, 66	≥95, 78	[28]
42a, b CH_3		NH_2	$[\alpha]_D^{25}$ −11.2, 78 (1.0, MeOH)		[28]

Table 11.1-7. (cont.).

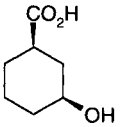
$CH_3-CH(OCOR^1)-CO_2R^2$ (±)

R^1	D-acid (R^2 = H) op (%), yield (%)	L-ester (R^2 = C_2H_5) op (%), yield (%)	Ref.
43a, b C_6H_5	high, 28	82, 88	[21]
44a, b CH_3	73, 85	73, 85	[21]



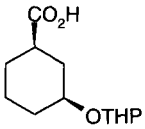
(±)

R^1	D-acid (R^2 = H) op (%), yield (%)	L-ester (R^2 = C_2H_5) op (%), yield (%)	Ref.
45a, b $NHCOCH_3$	84, 28	high, 93	[25]
46a, b OH	high, 100	high, 84	[29]



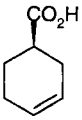
47 [30]

42% ee,



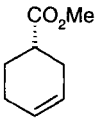
48 [30]

50% ee



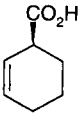
49a [30]

44% ee



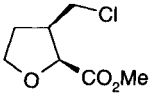
49b [30]

47% ee



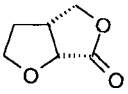
50 [30]

≥70% ee
50% conversion



51a [31]

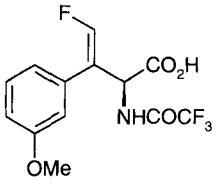
86% ee, 38% yield



51b [31]

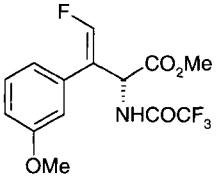
82% ee, 35% yield

Table 11.1-7. (cont.).



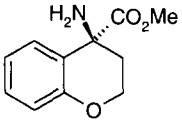
52a [32]

≥95 % ee, 71 % yield, DMF
no hydrolysis of
the Z-isomer



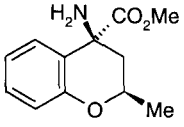
52b [32]

≥95 % ee, DMF



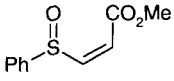
53 [33]

high op, 36 % yield



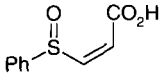
54 [33]

high op, 38 % yield



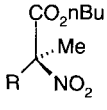
55a [34]

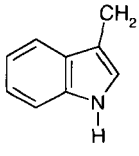
91 % ee, *t*BuOMe
58 % conversion
absolute configuration unknown



55b [34]

65 % ee, *t*BuOMe
58 % conversion
absolute configuration unknown



R = 

56 [35]

≥95 % ee, 27 % yield

R = Ph

57 [35]

≥95 % ee

58 [35]

≥95 % ee

59 [35]

85 % ee

$MeO_2C(CH_2)_2$
75 % ee

60 [35]

1 F. Björkling, J. Boutelje, S. Gatenbeck, K. Hult, T. Norin, P. Szmulik, *Tetrahedron* **1985**, *41*, 1347.
2 S. J. Cohen, E. Khedouri, *J. Am. Chem. Soc.* **1961**, *83*, 4228.
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19 Y. Hayashi, W. B. Lawson, *J. Biol. Chem.* **1969**, *244*, 4158.
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21 S. G. Cohen, Y. Sprinzak, E. Khedouri, *J. Am. Chem. Soc.* **1961**, *83*, 4225. S. G. Cohen, S. Y. Weinstein, *ibid* **1964**, *86*, 725.
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24 R. Chenevert, R. Leturneau, *Chem. Lett.* **1986**, 1151.
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31 J. H. Udding, J. Fraanje, K. Gaubitz, H. Hiemstra, W. N. Speckamp, K. Kaptein, H. E. Schoemaker, J. Kamphuis, *Tetrahedron: Asymmetry* **1993**, *4*, 425.
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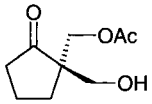
33 N. L. Dirlam, B. S. Moore, F. J. Urban, *J. Org. Chem.* **1987**, *52*, 3587.
34 C. Cardellicchio, F. Naso, A. Seilimati, *Tetrahedron Lett.* **1994**, *35*, 4635.
35 J. J. Calonde, D. E. Bergbreiter, D.-H. Wong, *J. Org. Chem.* **1988**, *53*, 2323.

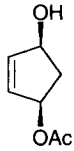
α-Chymotrypsin has been used most frequently and with much success for the enantiomer-differentiating hydrolysis of a wide range of natural and non-natural amino acid ester derivatives (Table 11.1-7), which usually leads in both cases to a mixture of the L-amino acid derivative and the D-amino acid ester derivative (**15–36**, **52**, and **53**). Excellent substrates for α-chymotrypsin are aromatic amino acid ester derivatives, but those amino acid esters which carry aliphatic or functionalized aliphatic chains of a certain length are excellent substrates also. Even a methyl or a nitro group as substituent is tolerated by the enzyme. Upon placement of a methylene group between the ester group and the stereogenic center, enantiomer differentiation is reverted and the D-acid derivative and the L-acid ester derivative are formed (**14** vs **45**). Interestingly, α-chymotrypsin also exhibits high enantiomer selectivity toward aromatic amino acids which bear a methyl group at the Cα-atom (**40–42**). Nitro analogs of methyl-substituted amino acids were also found to be suitable substrates for an α-chymotrypsin-catalyzed resolution (**56–60**). Enantiomer-differentiating hydrolysis of α-hydroxy acid ester derivatives is also feasible (**43** and **44**). As organic cosolvents, dimethyl sulfoxide, dimethylformamide and *tert*-butanol have been used without significant deactivation of the enzyme. The enantiomer

Table 11.1-8. Acetylcholine esterase-catalyzed enantiotopos-differentiating hydrolysis of prochiral cyclic diol diacetates and of racemic monoacetates in aqueous solution.



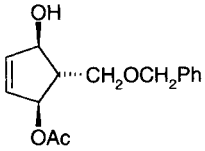
1 [1]

62% ee, 62% yield



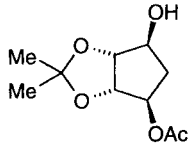
2 [2, 3]

96% ee, 93% yield



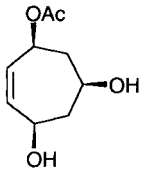
3 [4]

≥95% ee, 95% yield



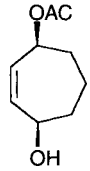
4 [5]

98% ee, 795 yield



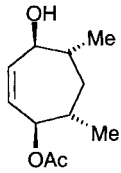
5 [6]

≥95% ee, 79% yield



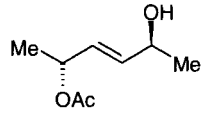
6 [7]

100% ee, 39% yield



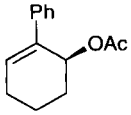
7 [7]

no hydrolysis



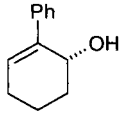
8 [8]

92% ee, 77% yield



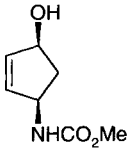
9a [9]

82% ee
50% conversion



9b [9]

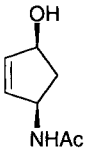
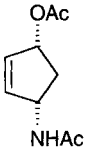
71% ee
50% conversion



10 [10]

72% ee,

Table 11.1-8. (cont.).

	**11a** [10]		**11b** [10]
92% ee,		92% ee, after a second hydrolysis	

1 H. Suemune, T. Harabe, Z. F. Xie, K. Sakai, *Chem. Pharm. Bull.* **1988**, *36*, 437.
2 D. R. Deardoff, A. J. Matthews, D. S. Mc Heekin, C. L. Carney, *Tetrahedron Lett.* **1986**, *27*, 1255.
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differentiation by α-chymotrypsin can rather accurately be explained by the active site model for the enzyme.

11.1.1.1.3 Acetylcholine Acetylhydrolases

Acetylcholine acetylhydrolase (E.C. 3.1.1.7) or acetylcholine esterase is a well characterized hydrolase[109] which is commercially available.

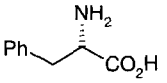
Acetylcholine esterase-catalyzed hydrolyses have been reported only for a small number of prochiral diacetates (Table 11.1-8). However, several of secondary monoacetates, which are valuable synthetic building blocks, have been obtained with high enantioselectivity (**2–6** and **11**) by using this enzyme. Acetylcholine esterase should be considered for the hydrolysis of diacetates which are not substrates for lipases and pig liver esterase.

Monoacetates of Table 11.1-8, which can be obtained with other hydrolases as such or of opposite configuration, are contained in Tables 11.1-3, 11.1-7 and 11.1-18.

11.1.1.1.4 Subtilisin

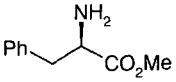
Subtilisins are a family of serine proteases, the most important members of which are subtilisin Carlsberg (from *Bacillus licheniformis*) and subtilisin BPN' (from *Bacillus amyloliquefaciens*)[110]. Both enzymes are alkaline proteases with a pH optimum of 6-9. Because of their industrial importance, both subtilisin Carlsberg and subtilisin BPP' have been studied intensively and are produced on a large scale. The crystal structures of both subtilisins have been determined[82]. Directed evolution and site-directed mutagenesis and chemical modification of subtilisin were carried out in order to influence the stability, activity and enantioselectivity of the enzyme, in particular in organic solvents[111]. As in the case of other enzymes,

Table 11.1-9. Subtilisin-catalyzed hydrolysis of racemic and prochiral esters.



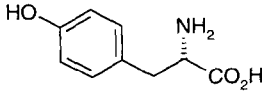
1a [1, 2]

90% ee, 96% yield



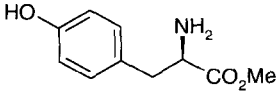
1b [1, 2]

100% ee, 85% yield



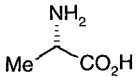
2a [1]

91% ee, 95% yield



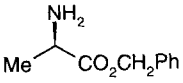
2b [1]

100% ee, 86% yield



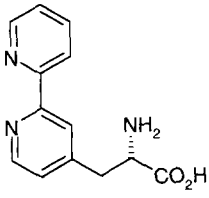
3a [1]

86% ee, 98% yield



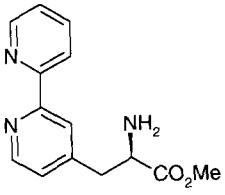
3b [1]

93% ee, 75% yield



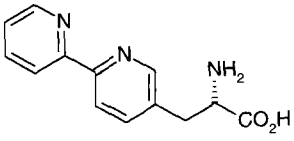
4a [3]

93% ee, 50% yield



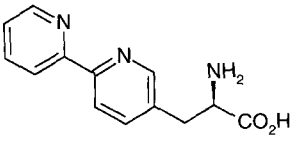
4b [3]

98% ee, 50% yield



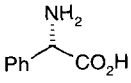
5a [3]

93% ee, 50% yield



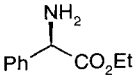
5b [3]

98% ee, 50% yield



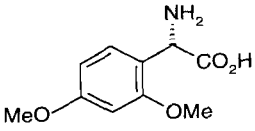
6a [4]

97% ee, 93% yield



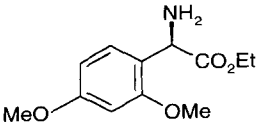
6b [4]

91% ee, 100% yield



7a [4]

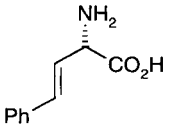
90% ee, 89% yield



7b [4]

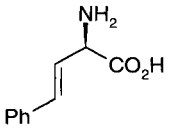
95% ee, 101% yield

Table 11.1-9. (cont.).



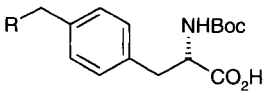
8a [4]

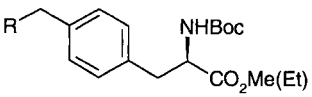
82 % ee, 100 % yield



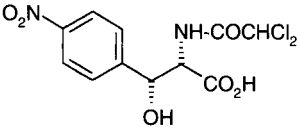
8 [4]

93 % ee, 95 % yield



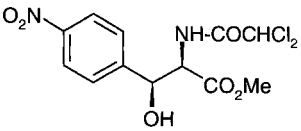


9a [5, 6] R = SO_3Na,	≥95 % ee, 47 % yield	**9b** [5, 6]	≥95 % ee, 48 % yield
10a [5, 6] R = $P(O)(OEt)_2$	≥95 % ee, 40 % yield	**10b** [5, 6]	≥95 % ee, 46 % yield
11a [5, 6] R = CO_2CH_2Ph,	≥95 % ee, 37 % yield	**11b** [5, 6]	≥95 % ee, 40 % yield
12a [5, 6] R = CO_2Me,	≥95 % ee, 49 % yield	**12b** [5, 6]	≥95 % ee, 47 % yield
13a [5, 6]R = $CONHCH_2Ph$,	≥95 % ee, 43 % yield	**13b** [5, 6]	≥95 % ee, 46 % yield



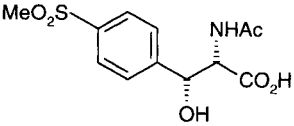
14a [7]

≥97 % ee, 96 % yield



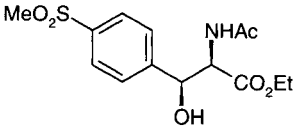
14b [7]

≥97 % ee, 99 % yield



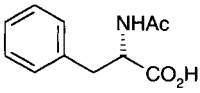
15a [8]

≥98 % ee
47 % conversion



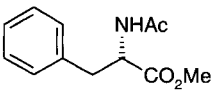
15b [8]

84 % ee
47 % conversion



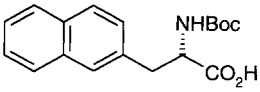
16a [8]

96 % ee
51 % conversion
CLEC-Subtilisin



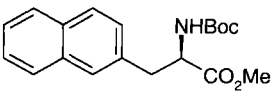
16b [8]

≥99 % ee
51 % conversion



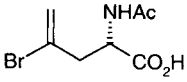
17a [8]

≥99 % ee
46 % conversion



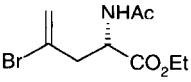
17b [8]

85 % ee
46 % conversion



18a [9]

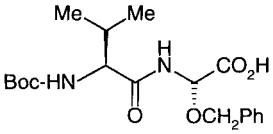
97 % ee,



18b [9]

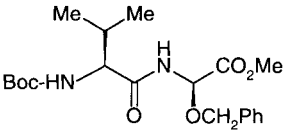
96 % ee

Table 11.1-9. (cont.).



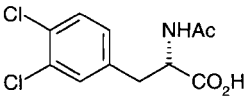
19a [10]

≥99% ee, 50% yield
starting from a 1:1 mixture



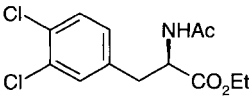
19b [10]

≥99% de, 50% yield
starting from a 1:1 mixture



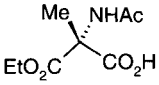
20a [11]

96% ee, 45% yield



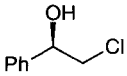
20b [11]

45% yield



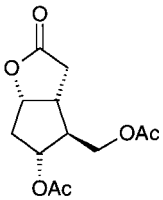
21 [12]

81% ee, ≥90% yield



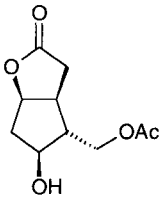
22 [13]

v_S/v_R = 6.8, dioxane



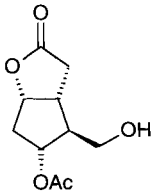
23a [14]

80% ee, 29% yield



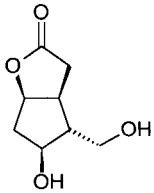
23b [14]

86% ee, 25% yield



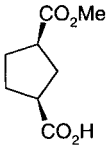
23c [14]

55% ee, 25% yield
acetone



23d [14]

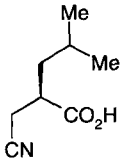
74% ee, 11% yield



24 [15]

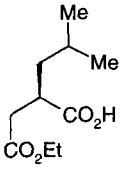
88% ee, 85% yield
90% ee, 95% yield, CLEC-subtilisin

Table 11.1-9. (cont.).



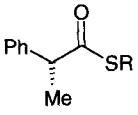
25 [16]

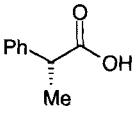
93 % ee
guanidinium chloride



26 [16]

99 % ee, 47 % yield



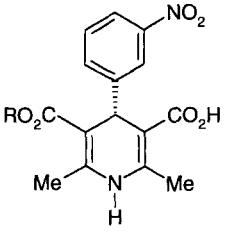


27 [17] R = $CH_2C{\equiv}CH$, 74 % ee, H_2O, MeCN
43 % conversion

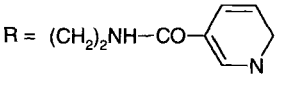
80 % ee, MeCN, H_2O, trioctyl amine
95 % conversion (with dynamic kinetic resolution)

28 [17] R = CH_2CF_3, 73 % ee, H_2O, MeCN
35 % conversion

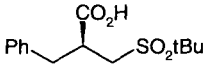
83 % ee, H_2O, MeCN, trioctyl amine
97 % conversion (with dynamic kinetic resolution)



29 [18]

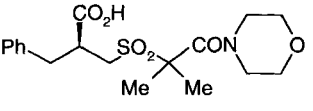


≥99 % ee, 41 % yield



30 [19]

≥98 % ee,



31 [19]

≥98 % ee,

1 S.-T. Chen, K.-T. Wang, C.-H. Wong, *J. Chem. Soc., Chem. Commun.* **1986**, 1514.
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CLECs of subtilisin have been prepared[112]. Autohydrolysis of subtilisin-CLECs seems to suppressed as compared to subtilisin. Subtilisin has been widely used for the kinetic resolution of amino acid esters. Hydrolyses catalyzed by subtilisin are usually carried out in aqueous buffer solution in a pH range of 7-8. In the case of a low solubility of the substrate in water, cosolvents such as methanol, ethanol, dimethylformamide or dimethyl sulfoxide may be used. Subtilisin is the hydrolase of choice for the racemate separation of natural and non-natural amino acid esters (**1–18** and **20**) (Table 11.1-9). Generally, the L-amino acid ester is preferentially hydrolyzed. Free amino acid esters as well as *N*-protected amino acid esters are substrates for subtilisin. The utility of subtilisin for the synthesis of enantioenriched amino acids is impressively demonstrated by the highly selective resolution of amino acid esters, the side chains of which contain functional groups (**9–15**). Frequently, subtilisin is preferred in the large scale resolution of amino acid esters rather than other hydrolases because of its lower price. Not only racemic amino acid esters but also other racemic carboxylic acid esters have been resolved with subtilisin. Impressive examples in terms of selectivity and efficiency are the hydrolyses yielding the functionalized esters **25**, **26**, and **29–31**, which were prepared on a large scale. Activity and selectivity of the enzyme in the kinetic resolution of the racemic esters of **30** and **31** could be improved by addition of dimethyl sulfoxide or guanidinium chloride, which was used at a concentration of 10 mM. A particularly interesting example of a kinetic resolution with subtilisin is that of the racemic thioesters derivatives of **27** and **28**. This was carried out in the presence of a base to a ensure a dynamic kinetic resolution (see Sect. 11.1.1.2.1.2) through base-catalyzed racemization of the non-hydrolyzed enantiomeric thioester.

11.1.1.1.5 Lipases

Lipases (triacyl glycerol acyl hydrolases, E.C. 3.1.1.3) are a unique class of hydrolases[113–115] for asymmetric synthesis based on prochiral or racemic substrates. The application of lipases as biocatalysts has been reviewed emphasizing different aspects in a number of books[9, 21, 22, 26, 28, 30, 32, 34, 35] and journals[11, 14, 18, 19, 24, 25, 27, 29, 31, 116, 117]. Lipases are catalytically active in water, in mixtures of water and water-immiscible or miscible organic solvents, in almost anhydrous organic solvents, and in supercritical fluids[34, 36] and ionic liquids[118, 119]. They are available from plants, mammals, and microorganisms in considerable numbers, which explains in part their versatility for asymmetric synthesis. Lipases are typical induced-fit enzymes, accepting non-natural substrates of enormous structural diversity.

There is some confusion in the literature regarding the origin and the name of

some microbial lipases. The lipase from *Pseudomonas cepacia* from Amano was formerly called lipase from *Pseudomonas fluorescens*, and was most recently re-identified as *Burkholderia cepacia* lipase. *Candida cylindracea* lipase was re-identified as *C. rugosa* lipase and *Mucor miehei* lipase was re-identified as *Rhizomucor miehei* lipase[34]. *Candida antarctica* produces the two lipases A and B that are/were available either as a mixture or in both individual forms. In order to avoid any further confusion in this text, by and large the names from the original papers have been used, but the special supplier names have been translated into names referring to the biological origin so far as unambiguously possible.

The lipases most used until now are the commercially supplied pig pancreas lipase (PPL), *Pseudomonas cepacia* lipase (PCL) or *P. fluorescens* lipase (PFL), *Candida cylindracea* (CCL) or *C. rugosa* lipase (CRL), *Pseudomonas* sp. lipase (PSL), increasingly *Candida antarctica* B lipase (CAL-B) and to a lesser extent further lipases mentioned in Tables 11.1-10 to 11.1-25, and cholesterol esterase (CE). CAL-B is a recombinant protein produced in *Aspergillus oryzae* accepting a broad range of substrates and conditions. A special group of hydrolases, which are considered as lipases, are the cholesterol esterases (CE), found in mammals and microorganisms[113].

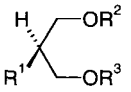
About 70 different lipases are commercially available. Most of these are presumably serine hydrolases containing a serine residue in their active site and featuring presumably the triad Ser ... His .... Asp. The crystal structures of the 13 different lipases have been determined[84-87].

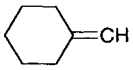
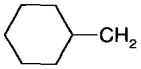
The molecular weight of the known lipases in their active, native form ranges from 30 to 65 kDa. Lipases are generally soluble in water and insoluble in organic solvents, and may be strongly adsorbed at the air/water interface.

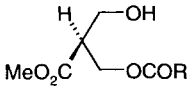
Lipases are available and applied as lyophilized powders, in covalently and non-covalently immobilized form on inorganic or organic carriers, in sol-gel material[120, 121] and as CLECs[64e, 122]. Most mammalian lipases exhibit pH optima ranging from 8 to 9 and most microbial lipases from 5.6 to 8.5. The temperature range for optimal activity is between 30 and 50 °C. In the case of labile substrates or insufficient enantiomer selectivity, hydrolysis may be carried out in water-saturated water-immiscible organic solvent such as diisopropyl ether, hexane or cyclohexane.

Most lipases are applied as crude materials consisting of a mixture of proteins that may even contain other hydrolases together with stabilizing solid supports. Pig pancreas lipase is a glycoprotein which exists as a mixture of isoenzymes differing in the glycan moiety of the enzyme. Crude pancreas lipase contains presumably another carboxyl esterase that may be responsible at least in part for the high enantioselectivity frequently observed with this enzyme in hydrolysis and esterification[44, 123-125]. Therefore, an isolated lipase of the same origin may have different activities and selectivities depending on the isolation and purification procedures of the individual suppliers. Some of these problems can be overcome, however, by the application of purified lipases, which are also commercially available. Lipases exhibit high catalytic activity in water and an even higher activity in two-phase systems composed of water and a water-immiscible organic solvent or water and a liquid substrate. In two-phase systems like water and *tert*-butyl methyl ether or water and

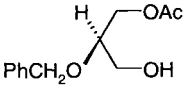
Table 11.1-10. Lipase-catalyzed enantiotopos-differentiating hydrolysis of prochiral acyclic diol diacetates in aqueous solution (MJL *Mucor javanicus* lipase, PFL *Pseudomonas fluorescens* lipase, PPL ig pancreas lipase, PCL *Pseudomonas cepacia* lipase).



	R^1	R^2	R^3	Lipase	ee (%)	yield (%)	Ref.
1	$(CH_3)_2CH$	H	Ac	PPL, crude	37	–	[1]
1	$(CH_3)_2CH$	H	Ac	PPL[a]	75	91	[1]
1	$(CH_3)_2CH$	H	Ac	PPL, pure	very slow	hydrolysis	[1]
2	CH_2Ph	H	Ac	PPL[a]	61	65	[1, 2]
3	$CH_2{=}CH{-}CH_2$	H	Ac	PPL	95	34	[4]
4	$CH_2{=}CH{-}(CH_2)_2$	H	Ac	PPL[a]	≥95	80	[2]
5	Ph	H	Ac	PPL[a]	≥95	91	[2]
5	Ph	H	Ac	PFL	94	41	[3]
6	c-C_6H_{11}	H	Ac	PPL[a]	60	96	[2]
7	(*E*)-*n*-Pent-CH=CH	H	Ac	PPL	84	49	[5, 6]
8	(*E*)-*n*-Pent-CH=CH	H	Ac	PPL[b]	95	63	[5, 6]
8	(*E*)-*n*-Pent-CH=CH	H	Ac	PPL[c]	93	59	[5, 6]
9	(*Z*)-*n*-Pent-CH=CH	Ac	H	PPL	50	43	[6]
9	(*Z*)-*n*-Pent-CH=CH	Ac	H	PPL[c]	53	31	[6]
9	(*Z*)-*n*-Pent-CH=CH	Ac	H	PPL[b]	55	44	[6]
10	(*E*)-*i*-Pr-CH=CH	H	Ac	PPL	90	70	[6]
10	(*E*)-*i*-Pr-CH=CH	H	Ac	PPL[c]	97	75	[6]
10	(*E*)-*i*-Pr-CH=CH	H	Ac	PPL[b]	88	71	[6]
11	(*Z*)-*i*-Pr-CH=CH	Ac	H	PPL	21	25	[6]
11	(*Z*)-*i*-Pr-CH=CH	Ac	H	PPL[c]	15	20	[6]
12	*n*-Hep	H	Ac	PPL[c]	70	56	[6]
13	*i*-Pent	H	Ac	PPL[c]	72	47	[6]
14	*n*-Pent-C≡C	H	Ac	PPL	78	57	[6]
				PPL[c]	80	50	[6]
				PPL[b]	82	61	[6]
15	*i*-Pr-C≡C	H	Ac	PPL	82	67	[6]
				PPL[c]	85	65	[6]
				PPL[b]	88	71	[6]
16		H	Ac	PPL	67	29	[6]
17		H	Ac	PPL	2	45	[6]



18



19

Table 11.1-10. (cont.).

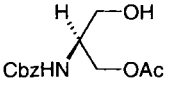
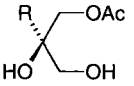
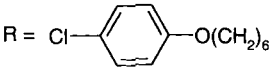
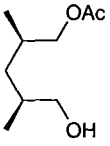
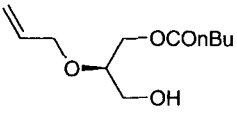
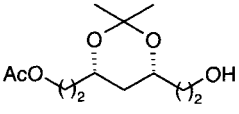
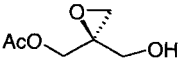
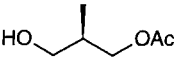
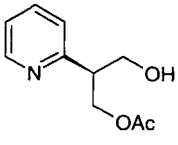
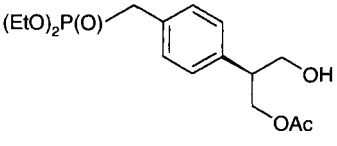
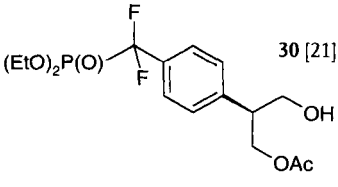
R	Ref.		Ref.
*n*Pr, 65 % ee, 29 % yield, PPL[d]	[7]	60 % ee, 40 % yield, PPL	[8]
*n*Bu, 68 % ee, 36 % yield, PPL[d]	[7]	80 % ee, 40 % yield, PPL[d]	[8]
nC_5H_{11}, 65 % ee, 29 % yield, PPL[d]	[7]	88 % ee, 45 % yield, PPL[c]	[8]
nC_6H_{13}, 70 % ee, <17 % yield, PPL[d]	[7]	91 % ee, 75 % yield, PFL	[8]
nC_7H_{15}, 84 % ee, 48 % yield, PPL[d,e]			
 **20** [10]		 **21** [10]	
		R = 	
≥97 % ee,55 % yield, PPL		73 % ee, 46 % yield, PPL 91 % ee, 75 % yield, PPL[g] R = ME-$(CH_2)_{13}$ 57 % ee, 66 % yield, PPL 87 % ee, 49 % yield, PPL[g]	
 **22** [12]		 **23** [13]	
89 % ee, 82 % yield, PPL		95 % ee, –, MJL	
 **24** [14, 15]		**25** [16]	
≥98 % ee, 98 % yield, PFL		97 % ee, 83 % yield, PFL	
 **26** [17, 18]		 **27** [19]	
96 % ee, 85 % yield, PFL		≥99 % ee, 33 % yield, PFL	
 **28** [20]		 **29** [21]	
78 % ee, 80 % yield, PPL[h]		98 % ee, yield 63 %, PCL[i]	
 **30** [21]			
		95 % ee, yield 65 %, PCL[i]	

Table 11.1-10. (cont.).

a Protein fraction from chromatography of crude PPL
b 10 % *tert*-BuOH
c Diisopropyl ether
d Absolute configuration unknown
e 30 % MeOH
f 15 % Tetrahydrofuran
g Hexane
h 15 % *t*-BuOH
i 30 % Diisopropyl ether

1 G. M. Ramos Tombo, H.-P. Schär, X. Fernandez I Busquets, O. Ghisalba, in: C. Laane, J. Tramper, M. D. Lilly (Eds.), *Biocatalysis in Organic Media*, p. 43, Elsevier, Amsterdam, **1987**.
2 G. M. Ramos Tombo, H.-P. Schär, X. Fernandez I Busquets, O. Ghisalba, *Tetrahedron Lett.* **1986**, *27*, 5707.
3 S. Atsumi, M. Nakano, Y. Koike, S. Tanaka, M. Ohkubo, T. Yonezawa, H. Funabashi, J. Hashimoto, H. Morishima, *Tetrahedron Lett.* **1990**, *31*, 1601.
4 Y.-F. Wang, C. J. Sih, *Tetrahedron Lett.* **1984**, *25*, 4999.
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6 G. Guanti, L. Banfi, E. Narisano, *Tetrahedron: Asymmetry* **1990**, *1*, 721.
7 J. Ehrler, D. Seebach, *Liebigs Ann. Chem.* **1990**, 379.
8 D. Breitgoff, K. Laumen, M. P. Schneider, *J. Chem. Soc., Chem. Commun.* **1986**, 1523.
9 V. Kerscher, W. Kreiser, *Tetrahedron Lett.* **1987**, *28*, 531.
10 Y.-F. Wang, J. J. Lalonde, M. Momongan, D. E. Bergbreiter, C.-H. Wong, *J. Am. Chem. Soc.* **1988**, *110*, 7200.
11 K. Prasad, H. Estermann, C.-P. Chen, O. Repic, G. E. Hardtmann, *Tetrahedron: Asymmetry* **1990**, *1*, 421.
12 Y.-F. Wang, C.-S. Chen, G. Girdaukas, C. J. Sih, *J. Am. Chem. Soc.* **1984**, *106*, 3695.
13 B. Wirz, R. Schmid, J. Foricher, *Tetrahedron: Asymmetry* **1992**, *3*, 137.
14 C. Bonini, R. Racioppi, L. Viggiani, G. Righi, L. Rossi, *Tetrahedron: Asymmetry* **1993**, *4*, 793.
15 C. Bonini, R. Racioppi, G. Righi, L. Viggiani, *J. Org. Chem.* **1993**, *58*, 802.
16 N. Adjé, O. Breuilles, D. Uguen, *Tetrahedron Lett.* **1993**, *34*, 4631.
17 T. Itoh, H. Ohara, Y. Takagi, N. Kanda, K, Uneyama, *Tetrahedron Lett.* **1993**, *34*, 4215.
18 Y.-B. Seu, Y.-H. Kho, *Tetrahedron Lett.* **1992**, *33*, 7015.
19 Z.-F. Xie, H. Suemune, K. Sakai, *Tetrahedron: Asymmetry* **1993**, *4*, 973.
20 G. Guanti, E. Narisano, R. Riva, *Tetrahedron: Asymmetry* **1997**, *8*, 2175.
21 T. Yokomatsu, T. Minowa, T. Murano, S. Shibuya, *Tetrahedron* **1998**, *54*, 9341.

hexane, much higher reaction rates and enantioselectivities are most frequently observed. A synthetically useful alternative to the lipase-catalyzed hydrolysis of an ester in aqueous solution for the attainment of a chiral carboxylic acid or alcohol is the lipase-catalyzed alcoholysis of an ester described in Sect. 11.1.1.3.

Generally, through (a) hydrolysis or alcoholysis of a prochiral dialkanoate or racemic alkanoate by water in homogeneous and heterogeneous aqueous/organic mixtures or by an alcohol in an organic solvent and (b) acylation of the corresponding prochiral diol or racemic alcohol in an organic solvent, access to both enantiomers of the corresponding monoacetate and alcohol, respectively, is provided with one enzyme [(a) Tables 11.1-10 to 11.1-12, 11.1-14 to 11.1-16, and 11.1-22; (b) Tables 11.1-17 to 11.1-21] (Scheme 11.1-12). Hydrolysis and ester formation are enantiocomplementary, which means that one and the same lipase for a given substrate reacts preferentially with the same enantiotopic group or the same enantiomer irrespective of whether it is an ester or an alcohol. Enantioselectivities and yields may differ in hydrolysis and alcoholysis, respectively, and in acylation. A combination of these routes may be advantageous in a given case for the attainment of both enantiomers in high enantiomeric purity (Schemes 11.1-11 and 11.1-14). One cannot expect, however, that a lipase will transform a non-natural substrate in an optimal manner. Therefore, different, more or less empirical, strategies have been

developed to improve reactivity and/or selectivity. The substrate structure and the origin of the lipase mainly determine reactivity and selectivity in a lipase-catalyzed reaction. Hence, the first step of an envisaged lipase-catalyzed kinetic resolution or desymmetrization of a given substrate comprises rapid screening of available lipases [35e]. There is no simple relationship, however, between structural parameters of a given substrate, the origin of the lipase and the outcome of the reaction. On the other hand, models have been developed in order to understand and predict the behavior of certain lipases toward structural properties of substrates with regard to reactivity and selectivity [57, 74-79, 126-132] that help to optimize the reaction by modifying the substrate structure. As mentioned before, lipase activity and selectivity are strongly influenced by the medium used for the desired reaction. Having identified a suitable lipase, variation of the medium – solvent engineering – might be the next step in order to optimize the outcome of the reaction. An additional fine tuning of the reaction conditions is achievable by using certain additives, which may have a beneficial influence on the microenvironment of the lipases and hence on their selectivity and/or reactivity [133]. Finally, an increase of the selectivity of the lipase may be gained by lowering the temperature of the reaction mixture.

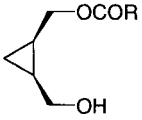
Primary acyclic diacetates are substrates par excellence for lipases (**1–24**, **26–30**) (Table 11.1-10). Prochiral 1,3-propane diol derivatives have been most thoroughly studied in terms of the influence of the substrate structure, the composition of the reaction medium and the origin and purity of the lipase on the enantioselectivity. A comparison of the hydrolysis of 2-alkyl substituted 1,3-propane diol diacetates with crude pig pancreas lipase, highly purified commercial pig pancreas lipase and a carboxyl esterase fraction isolated from crude pig pancreas lipase showed that the latter gave the monoacetate **1** with higher enantioselectivity and reaction rate than the first two (Table 11.1-10). Imitating the *in vivo* conditions for the action of lipase on triglycerides by addition of diisopropyl ether to the aqueous solution and carrying out the hydrolysis in the two-phase system leads in some but not all cases to a higher enantioselectivity and reaction rate, as demonstrated for the monoacetates **7** and **8**.

Unsaturation in the alkyl chain frequently leads to the monoacetate of a higher *ee* value as exemplified with **16** and **17**. Comparison of the enantioselectivities of the hydrolysis of diacetates to the corresponding monoacetates is often complicated by the lack of information on the amount of diol formed. The later arises from the hydrolysis of the monoacetate that may proceed under enantiomer differentiation, and thus the *ee* value of the monoacetate will be a composite of two enantioselective processes. Interestingly, upon changing the configuration of the double bond of the substituent R from (*E*) to (*Z*) the enantiotopic group recognition by pig pancreas lipase inverts, as demonstrated by the monoacetates **8** and **9** as well as **11** and **12** (Table 11.1-10).

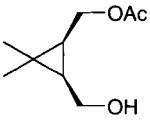
Group-selective and enantioselective hydrolyses as well as the influence of the alkyl group of the acyl function and the cosolvent upon the enantioselectivity are demonstrated by the pig pancreas lipase-catalyzed hydrolysis of the methoxycarbonyl substituted diacylated propanediol corresponding to the monoesters **18**.

Protected glycerol monoacetate **19** can be prepared in acceptable enantiomeric purity and yield by crude pig pancreas lipase or *Pseudomonas fluorescens* lipase-

Table 11.1-11. Lipase-catalyzed enantiotopos-differentiating hydrolysis of prochiral cyclic diol dialkanoates in aqueous solution (CCL *Candida cylindracea* lipase, PFL *Pseudomonas fluorescens* lipase, MML *Mucor miehei* lipase, CVL *Chromobacterium viscosum* lipase, PPL pig pancreas lipase, MJL *Mucor javanicus* lipase, RSL *Rhizopus* sp. lipase, PCL *Pseudomonas cepacia* lipase, GCL, *Geotricum candidum* lipase, ANL *Aspergillus niger* lipase, FSPC *Fusarium solani pisi* cutinase, CRL *Candida rugosa* lipase, CAL-B *Candida antarctica* B lipase, LIP *Pseudomonas* sp. lipase-Toyobo, RDL *Rhizopus delemar* lipase, MSL *Mucor* sp. lipase, CAL *Candida antarctica* lipase, not specified).



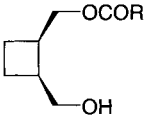
1



2 [1]

40 % ee, 75 % yield, PPL

R		
Me	72 % ee, 94 % yield, PPL	[1]
	93 % ee, 74 % yield, PPL	[2]
	90 % ee, 83 % yield, PFL	[3]
n-Pr	94 % ee, 84 % yield, PPL	[2]
	≥99 % ee, 100 % yield, PPL	[4]



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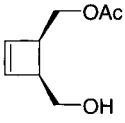
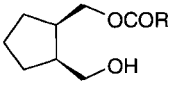


4

R				
Me	88 % ee, 94 % yield, PPL	[1]	86 % ee, 57 % yield, PPL	[6]
	96 % ee, 78 % yield, PPL	[2]	≥97 % ee, 75 % yield, PFL	[7]
	≥95 % ee, 87 % yield, PFL	[3]		
n-Pr	96 % ee, 80 % yield, PPL	[1]		



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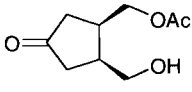
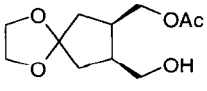


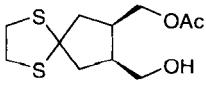
6

R				
Me	88 % ee, 94 % yield, PPL	[1]	50 % ee, 70 % yield, PPL	[5]
	89 % ee, 90 % yield, PPL	[2]	77 % ee, 78 % yield, PPL	[6]
	≥95 % ee, 86 % yield, PFL	[3]	(purified PPL)	
n-Pr	96 % ee, 80 % yield, PPL	[1]		



7 [5]

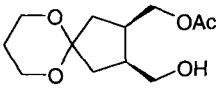
60 % ee, 75 % yield, PPL



8 [5]

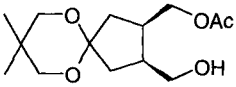
50 % ee, 71 % yield, PPL

Table 11.1-11. (cont.).



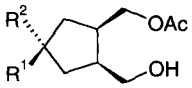
9 [5]

78 % ee, 77 % yield, PPL
93 % ee, 86 % yield, PPL
(purified PPL)



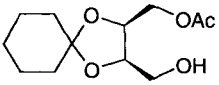
10 [5]

30 % ee, 75 % yield, PPL



11 [5, 6]

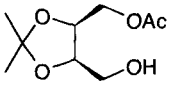
R^1	R^2	
HO	H	68 % ee, 80 yield, PPL
O-*t*-Bu	H	26 % ee, 80 yield, PPL
OMe	H	52 % ee, 65 yield, PPL
OEt	H	66 % ee, 78 yield, PPL
H	OAc	90 % ee, 60 yield, PPL
H	C1	88 % ee, 81 yield, PPL
H	C1	95 % ee, 88 yield, PPL
H	SPh	96 % ee, 65 yield, PPL
H	SO_2Ph	68 % ee, 72 yield, PPL
H	N_3	91 % ee, 85 yield, PPL



11 [7]

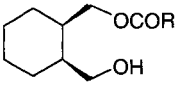
41 % ee, 73 % yield, PPL
81 % ee, 85 % yield, PPL
98 % ee, 91 % yield, PFL

(purified PPL)



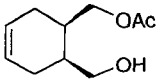
13 [7–9]

13 % ee, 70 % yield, PPL
0 % ee, –, PFL
97 % ee, 88 yield, PFL



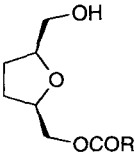
14

R		
Me	78 % ee, 81 yield, PPL	[1]
	87 % ee, 67 yield, PPL	[2]
	50 % ee, 54 yield, PPL	[3]
n-Pr	84 % ee, 24 yield, PPL	[2]



15

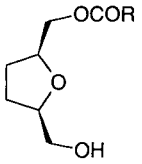
≥98 % ee, 96 % yield, PPL [1, 10]
92 % ee, 90 % yield, PFL [3]



16 [20]

R	
Me	41 % ee, 35 % yield, PPL
Et	33 % ee, 57 % yield, PPL
i-Pr	32 % ee, 68 % yield, PPL
*t*Bu	16 % ee, 71 % yield, PPL

Table 11.1-11. (cont.).



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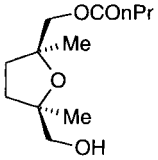
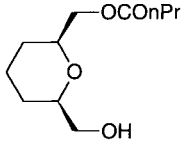


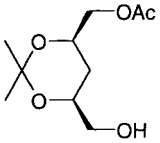
18 [12]

R			
Me	80 % ee, 20 % yield, CCL	[11]	99 % ee, 84 % yield, PFL
Et	26 % ee, 69 % yield, CCL	[11]	99 % ee, 66 % yield, MJL
i-Pr	28 % ee, 75 % yield, CCL	[11]	85 % ee, 50 % yield, PPL
t-Bu	4 % ee, 71 % yield, CCL	[11]	75 % ee, 37 % yield, CCL
n-Bu	≥99 % ee, 75 % yield, MJL	[12]	
	68 % ee, 85 % yield, CCL	[12]	



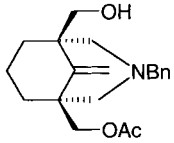
19 [13]

55 % ee, 77 % yield, PPL



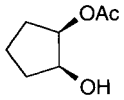
20 [8, 14]

96 % ee, 79 % yield, PFL



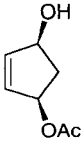
21 [15]

83 % ee, 26 % yield, CCL

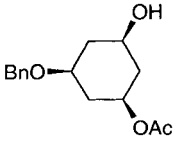


22 [16–18]

≥99 % ee, 39 % yield, PFL



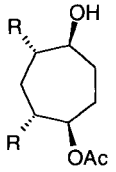
23



24 [23]

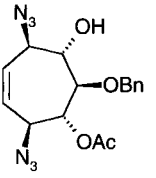
50 % ee, 82 % yield, CCL	[19]	36 % ee, 21 % yield, ANL	
66 % ee, 83 % yield, RSL	[19]	18 % ee, 13 % yield, PFL	
92 % ee, 87 % yield, PPL	[20]	84 % ee, 16 % yield, PFL	
92 % ee, 84 % yield, PFL	[21]		
95 % ee, 85 % yield, MML	[20]		
91 % ee, 76 % yield, CVL	[20]		
>99 % ee, 85 % yield, MSL	[22a]		
99 % ee, 90 % yield, CAL-B	[22b]		

Table 11.1-11. (cont.).



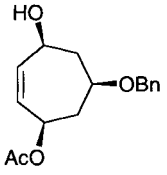
25 [24]

R
H 44 % ee, 40 % yield, CCL
Me 100 % ee, 61 % yield, CCL



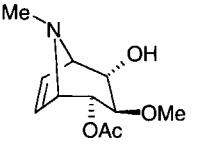
26 [25]

≥97 % ee, –, GCL



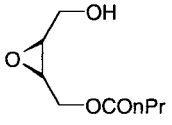
27 [25]

≥97 % ee, 60 % yield, PCL



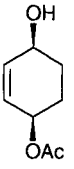
28 [25]

≥98 % ee, 100 % yield, ANL



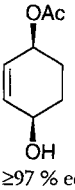
29 [26, 27]

95 % ee, 90 % yield, PPL



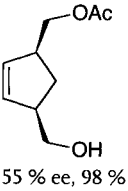
30 [28]

79 % ee, 64 % yield, PCL



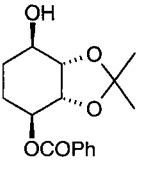
***ent*-30** [29]

≥97 % ee, –, CRL



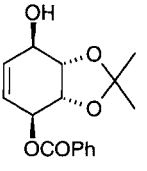
31 [30, 31]

55 % ee, 98 % yield, PPL
30 % ee, 45 % yield, CCL



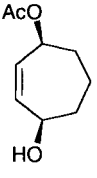
32 [32]

≥95 % ee, 94 % yield, FSPC



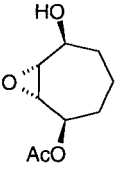
33 [32]

≥95 % ee, 87 % yield, FSPC



34 [33]

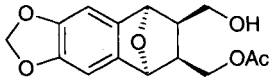
35 % ee, –, PCL



35 [33]

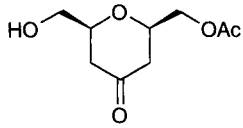
≥98 % ee, 89 % yield, PCL

Table 11.1-11. (cont.).



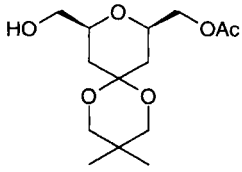
36 [34]

95 % ee, 66 % yield, PPL



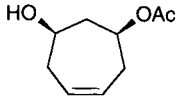
37 [35]

70 % ee, 80 % yield, PCL



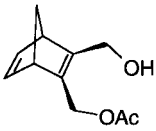
38 [35]

>98 % ee, 88 % yield, PCL



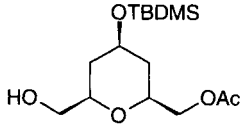
39 [36]

96 % ee, 72 % yield, PFL



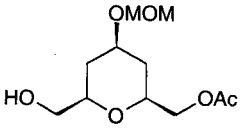
40 [37]

>95 % ee, 89 % yield, PSL



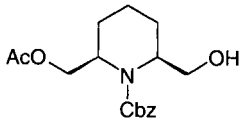
41 [38]

>95 % ee, 70 % yield, CAL



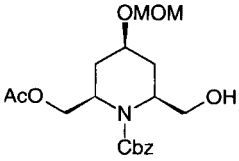
42 [38]

86 % ee, 100 % yield, CAL



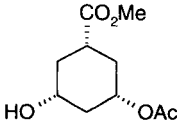
43 [39]

>98 % ee, 82 % yield, ANL



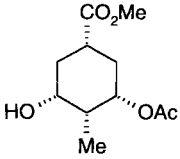
44 [39]

>98 % ee, 76 % yield, ANL



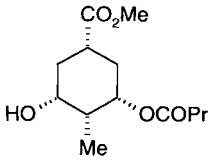
45 [40]

77 % ee, 39 % yield, PFL



46 [40]

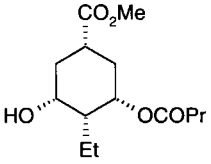
88 % ee, 49 % yield, PFL



47 [40]

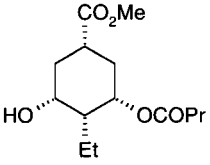
>99 % ee, 89 % yield, PSL

Table 11.1-11. (cont.).



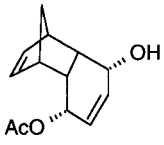
48 [40]

>99 % ee, 88 % yield, PFL



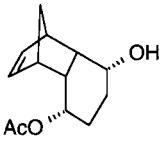
48 [40]

87 % ee, 49 % yield, PPL



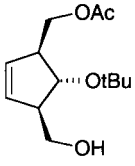
49 [41]

>99 % ee, 53 % yield, LIP



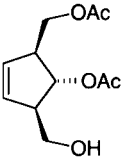
50 [41]

>99 % ee, 51 % yield, LIP



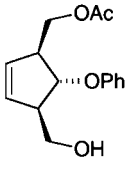
51 [42]

>99 % ee, 95 % yield, RDL
>99 % ee, 61 % yield, PFL
94 % ee, 60 % yield, PPL



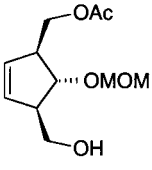
52 [42]

95 % ee, 86 % yield, RDL
33 % ee, 73 % yield, PFL



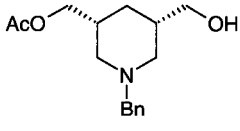
53 [42]

95 % ee, 64 % yield, RDL
87 % ee, 39 % yield, PPL



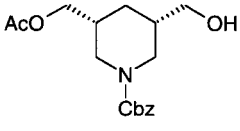
54 [42]

95 % ee, 95 % yield, RDL
16 % ee, 60 % yield, PPL



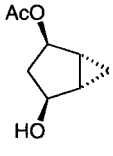
55 [43]

>98 % ee, 73 % yield, PFL



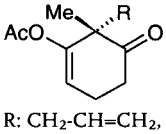
56 [43]

>98 % ee, 77 % yield, PFL



57 [44]

>99 % ee, 70 % yield, CAL-B



58 [45]

R: CH_2-CH=CH_2, CH_2-CH=CHCH_3(*E*), CH_2-CH=C(Cl)CH_3(*E*), CH_2-C≡C-CH_3, CH_2-Ph
>98 % ee, 62–80 % yield, CCL

40 Y. Zhao, Y. Wu, P. De Clercq, M. Vandewalle, P. Maillos, J.-C. Pascal, *Tetrahedron: Asymmetry* **2000**, *11*, 3887–3900.
41 H. Konno, K. Ogasawara, *Synthesis* **1999**, 1135.
42 M. Tanaka, Y. Norimine, T. Fujita, H. Suemune, K, Sakai, *J. Org. Chem.* **1996**, *61*, 6952.
43 B. Danieli, G. Lesma, D. Passarella, A. Silvani, *J. Org. Chem.* **1998**, *63*, 3492.
44 F. Theil, S. Ballschuh, M. von Janta-Lipinski, R. A. Johnson, *J. Chem. Soc., Perkin Trans.* 1 **1996**, 255.
45 P. Renouf, J.-M. Poirier, P. Duhamel, *J. Chem. Soc., Perkin Trans.* 1 **1997**, 1739.
46 T. Taniguchi, R. M. Kanada, K. Ogasawara, *Tetrahedron: Asymmetry* **1997**, *8*, 2773.
47 K. Toyama, S. Iguchi, T. Oishi, M. Hirama, *Synlett*, **1995**, 1243.

catalyzed hydrolysis of the corresponding diacetate. Here, too, enantioselectivity of the hydrolysis by crude pig pancreas lipase is considerably improved if the reaction is run in the two-phase system composed of water and diisopropyl ether.

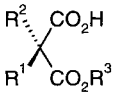
Glycerol diacetate derivatives with chain type substituent in the 2-position are hydrolyzed with crude pig pancreas lipase in a two-phase system composed of water and hexane to the monoacetates **21** with good enantioselectivity. Hydrolysis in aqueous solution alone is much less selective. The 2-benzyloxycarbonylamino-substituted propanediol monoacetate **20** is also accessible with a high *ee* value by pig pancreas lipase-catalyzed hydrolysis. Monoacetates **23–27** can serve as a good illustration of the scope of lipases because of the number of different species available. The monoacetate **24** is a notable example since it documents the surprising ability of *Pseudomonas fluorescens* lipase to differentiate between enantiotopic groups located relatively far from the stereogenic ring atoms. Monoacetates **27** and **29** are of opposite configuration compared to those obtained from the same achiral diacetates via pig liver esterase-catalyzed hydrolysis (Table 11.1-3).

Monoacetates of Table 11.1-10 which can be obtained with other hydrolases as such or of opposite configuration are contained in Tables 11.1-4 and 11.1-17.

One of the most successful applications of lipases lies in the hydrolysis of cyclic *meso*-configured dialkanoates, mainly diacetates, to the corresponding chiral monoalkanoates (**1–61**) (Table 11.1-11). However, the attainment of high enantioselectivity is not restricted to primary dialkanoates. Cyclic secondary dialkanoates are good substrates too (Table 11.1-11). There seems to be no restriction in regard to the ring size. Heterocyclic systems are tolerated by the various lipases. Reversal of enantiotopic group recognition in a series of structurally closely related substrates as frequently observed in the case of pig liver esterase-catalyzed hydrolysis is usually not observed with a lipase. This is illustrated by the series of cyclopentanoid dimethanol diacetates **7–12**. Enantioselectivity can be enhanced in many cases with a given lipase by either resorting to hydrolysis in a two-phase system, addition of a cosolvent, or changing the nature of the acyl group (**17**). If these measures fail, resorting to another lipase may lead to success. This is exemplified by the cyclopentenoid monoacetate **23**, which is obtained by *Candida cylindracea* lipase with an *ee* value of 50 %, by *Pseudomonas fluorescens* lipase with an *ee* value of 92 % and by *Mucor* sp. lipase or by *Candida antarctica* B lipase with *ee* values of ≥ 99 %. A frequently encountered synthetically very attractive situation is illustrated by the synthesis of the enantiomeric monoacetates **30** and ***ent*-30**. The two enantiomers are accessible with two different lipases.

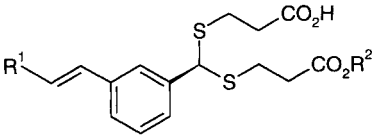
Tetrahydropyran derivatives **37**, **38**, **41** and **42** as well as the piperidine derivatives **44**, **45**, **55** and **56** can be prepared with high enantiomeric purity. Bi- and tricyclic

Table 11.1-12. Lipase-catalyzed enantiotopos-differentiating hydrolysis of prochiral acyclic and cyclic dicarboxylic acid diesters in aqueous solution (CCL *Candida cylindracea* lipase, PPL pig pancreas lipase, PSL *Pseudomonas* sp. lipase, CVL *Chromobacterium viscosum* lipase, CE cholesterol esterase).

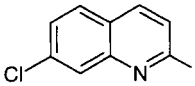


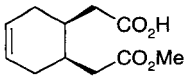
	R^1	R^2	R^3	Lipase	ee (%)	yield (%)	Ref.
1	CF_3	H	Me	CCl	no hydrolysis	–	[1]
2	F	Et	Me	CCL	99[a]	87	[1]
3	F	Me	Me	CCl	95	74	[1]
4	F	Me	Et	CCL	91	87	[2]
5	F	H	Et	PPL	61	23	[2]
5	F	H	Et	CCL	62[a]	70	[2]
6	F	Et	Et	CCL	93[a]	87	[2]
7	F	*n*-Pr	Et	CCL	33[a]	30	[2]
8	F	*n*-Bu	Et	CCL	11[a]	78	[2]

a Absolute configuration not determined



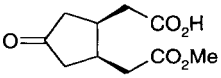
9

R^1	R^2		
	Me	≥98 % ee, 90 % yield, PSL, CVL	[3]
	CH_2CONEt_2	≥98 % ee, 90 % yield, CCL	[3, 4]



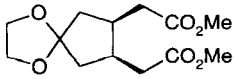
10 [5]

97 % ee, 97 % yield, PPL



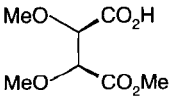
11 [5]

6 % ee, 48 % yield, PPL



12 [5]

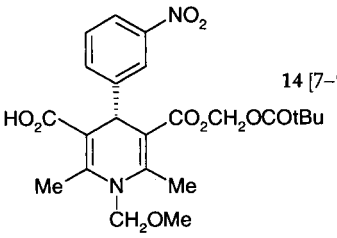
PPL, no hydrolysis



13 [6]

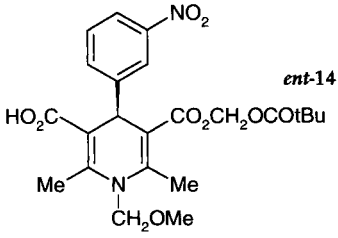
92 % ee, 90 % yield, CCL

Table 11.1-12. (cont.).



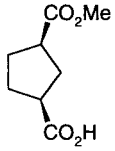
14 [7–9]

≥99 % ee, 80 % yield, lipase B, diisopropyl ether/H_2O
≥99 % ee, 80 % yield, PSL, diisopropyl ether/H_2O



ent-14 [9]

89 % ee, 87 % yield, PSL, diisopropyl ether/H_2O



15 [10]

90 % ee, 95 % yield, CE

1 T. Kitazume, T. Sato, N. Ishikawa, *Chem. Lett.* **1984**, 1811.
2 T. Kitazume, T. Sato, T. Kobayashi, J. Tain Lin, *J. Org. Chem.* **1986**, *51*, 1003.
3 D. L. Hughes, J. J. Bergan, J. S. Amato, P. J. Reider, E. J. J. Grabowski, *J. Org. Chem.* **1989**, *54*, 1787.
4 D. L. Hughes, Z. Song, G. B. Smith, J. J. Bergan, G. C. Dezeny, E. J. J. Grabowski, P. J. Reider, *Tetrahedron: Asymmetry* **1993**, *4*, 865.
5 Y. Nagao, M. Kume, R. C. Wakabayashi, T. Nakamura, M. Ochiai, *Chem. Lett.* **1989**, 239.
6 H. J. Bestmann, U. C. Philipp, *Angew. Chem.* **1991**, *103*, 78; *Angew. Chem. Int., Ed. Engl.* **1991**, *30*, 86.
7 H. Ebiike, Y. Terao, K. Achiwa, *Tetrahedron Lett.* **1991**, *32*, 5805.
8 H. Ebiike, K. Maruyama, K. Achiwa, *Tetrahedron: Asymmetry* **1992**, *3*, 1153.
9 Y. Hirose, K. Kariya, J. Sasaki, Y. Kurono, H. Ebiike, K. Achiwa, *Tetrahedron Lett.* **1992**, *33*, 7157.
10 R. Chenevert, R. Martin, *Tetrahedron: Asymmetry* **1992**, *3*, 199.

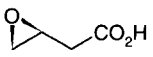
derivatives such as **36**, **40**, **49**, and **50** are obtained from the corresponding *meso*-diacetates.

The monoacetates **58**, **59** and **60** (Table 11.1-11) are products of the hydrolysis of prochiral enol diacetates.

Monoalkanoates of Table 11.1-11 which can be obtained with other hydrolases as such or of opposite configuration are contained in Tables 11.1-3, 11.1-7, 11.1-9 and 11.1-18.

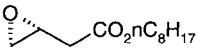
A limited number of acyclic and cyclic prochiral dicarboxylic acid diesters were found to be good substrates for hydrolysis catalyzed by lipases (Table 11.1-12). Notable examples which give a good illustration of the potential of hydrolases as well as of the trial and error approach one relies on to a certain extent are the dithio acetal derivative **9** and the fluoro alkyl malonates **1–8**. The dithio monoester **9** is obtained with different lipases with high enantioselectivities and yields despite its remote chiral center. *Candida cylindracea* lipase is the enzyme of choice for the synthesis of fluoro alkyl malonates with small alkyl groups. An astonishing observation was

Table 11.1-13. Lipase-catalyzed enantiomer-differentiating hydrolysis of racemic carboxylic acid esters and lactones in aqueous solution (PPL pig pancreas lipase, PSL *Pseudomonas* sp lipase, PFL *Pseudomonas fluorescens* lipase, CCL *Candida cylindracea* lipase, ANL *Aspergillus niger* lipase, PCL *Pseudomonas cepacia* lipase, CAL-A *Candida antarctica* A lipase, CRL *Candida rugosa* lipase, CAL *Candida antarctica* lipase, not specified).



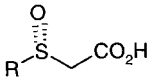
1a [1]

–, – PPL, 60 % conversion



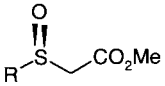
1b [1]

≥95 % ee, –



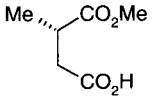
2a [2]

R = Ph, p-$NO_2C_6H_4$, p-$MeOC_6H_4$, $PhCH_2$, c-C_6H_{11}
80–100 % ee, 20–30 % yield, PSL
50 % conversion



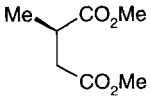
2b [2]

≥98 % ee, 30–50 % yield



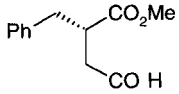
3a [3, 4]

95 % ee, 47 % yield, PPL
50 % conversion



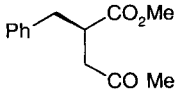
3b [3, 4]

≥96 % ee, –



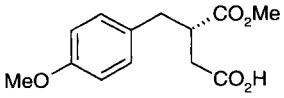
4a [3]

98 % ee, 45 % yield, PPL
50 % conversion



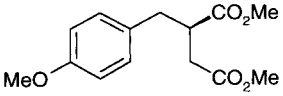
4b [3]

–, –



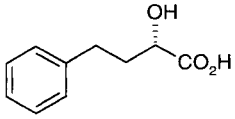
5a [5]

95 % ee, 40 % yield, PSL
42 % conversion



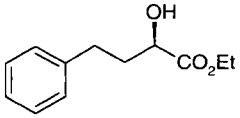
5b [5]

65 % ee, 55 % yield



6a [6]

92 % ee, 33 % yield, PFL
35 % conversion



6b [6]

99 % ee, 43 % yield
55 % conversion

Table 11.1-13. (cont.).

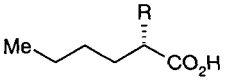
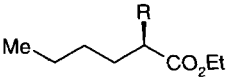
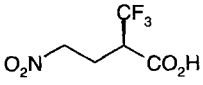
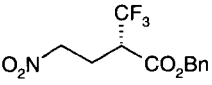
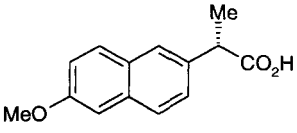
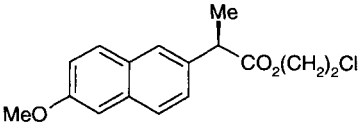
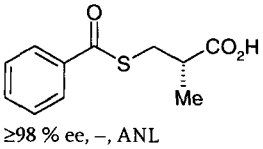
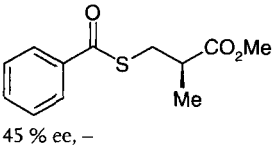
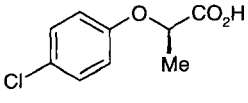
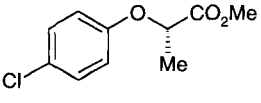
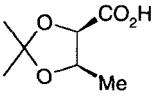
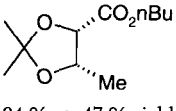
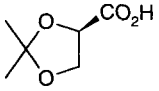
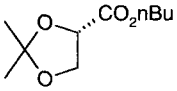
			
R = OH: 79 % ee, 48 % yield, PFL 50 % conversion	**7a**	95 % ee, 43 % yield	**7b** [6]
R = F: 69 % ee, 53 % yield, PFL 60 % conversion	**8a**	≥99 % ee, 37 % yield	**8b** [6]
R = Br: 69 % ee, 48 % yield, PFL	**9a**	73 % ee, 40 % yield	**9b** [6]
	**10a** [7]		**10b** [7]
≥98 % ee, –, PFL, 35 % conversion		98 % ee, –	
	**11a** [8]		**11b** [8]
98 % ee, –, CCL, 39 % conversion		63 % ee, –	
	**12a** [9]		**12b** [9]
≥98 % ee, –, ANL		45 % ee, –	
	**13a** [10–15]		**13b** [10–15]
96 % ee, 43 % yield, CCL 89 % ee, 49 % yield, PPL 93 % ee, 31 % yield, CRL	[16]	94 % ee, 48 % yield ≥99 % ee, 49 % yield 94 % ee, 46 % yield	[16]
	**14a** [17]		**14b** [17]
93 % ee, 35 % yield, CCL 50 % conversion		94 % ee, 47 % yield	

Table 11.1-13. (cont.).



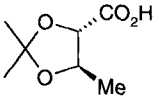
15a [17]

71 % ee, 53 % yield, PPL
57 % conversion



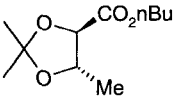
15b [17]

≥95 % ee, 40 % yield



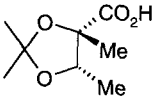
16a [17]

42 % ee, 38 % yield, CCL
69 % conversion



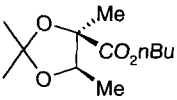
16b [17]

95 % ee, 19 % yield



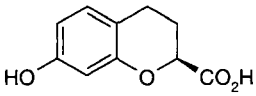
17a [17]

95 % ee, 41 % yield, CCL
55 % conversion



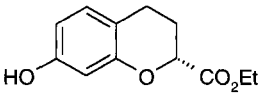
17b [17]

77 % ee, 35 % yield



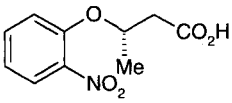
18a [6]

75 % ee, 50 % yield, PFL
55 % conversion



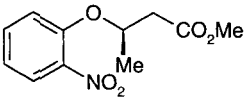
18b [6]

≥99 % ee, 45 % yield



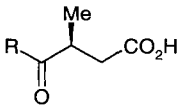
19a [18]

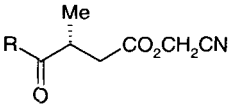
–, –, PCL
55 % conversion



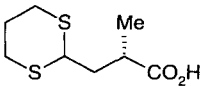
19b [18]

90 % ee, 52 % yield



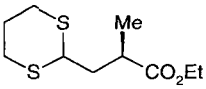


R				
Et	80 % ee, 49 % yield, PPL	**20a**	≥93 % ee, 44 % yield	**20b** [19]
C_5H_{11}	85 % ee, 49 % yield, PPL	**21a**	≥98 % ee, 49 % yield	**21b** [19]
$PhCH_2$	85 % ee, 49 % yield, PPL	**22a**	≥95 % ee, 49 % yield	**22b** [19]



23a [20]

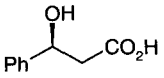
≥99 % ee, –, PPL



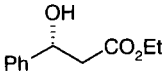
23b [20]

≥e99 % ee, –

Table 11.1-13. (cont.).



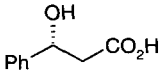
24a [21]



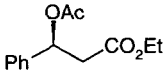
24b [21]

93 % ee, –, PSL

98 % ee, –



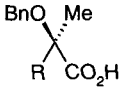
25a [21]

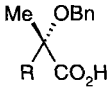


25b [21]

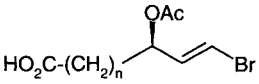
94 % ee, –, PSL

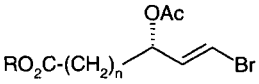
96 % ee, –



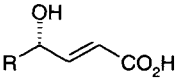


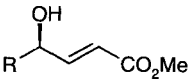
R				
Et	81 % ee, 41 % yield, CCL	**26a**	60 % ee, 54 % yield	**26b** [22]
n-Pr	95 % ee, 40 % yield, CCL	**27a**	70 % ee, 18 % yield	**27b** [22]
allyl	≥99 % ee, 46 % yield, CCL	**28a**	82 % ee, 52 % yield	**28b** [22]
n-C_6H_{13}	≥99 % ee, 38 % yield, CCL	**29a**	67 % ee, 35 % yield	**29b** [22]
n-C_9H_{19}	94 % ee, 46 % yield, CCL	**30a**	67 % ee, 8 % yield	**30b** [22]



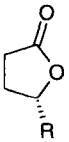


R	n				
Me	4	28 % ee, –, CCL	**31a**	–, –	**31b** [23]
Me	8	68 % ee, –, CCL	**32a**	–, –	**32b** [23]
n-Bu	8	≥99 % ee, –, CCL	**33a**	–, –	**33b** [23]





R				
n-Pr	84 % ee, 39 % yield, PPL	**34a**	75 % ee, 47 % yield	**34b** [24]
n-Bu	82 % ee, 40 % yield, PPL	**35a**	77 % ee, 46 % yield	**35b** [24]
n-C_5H_{11}	74 % ee, 50 % yield, PPL	**36a**	95 % ee, 40 % yield	**36b** [24]
n-C_6H_{13}	82 % ee, 48 % yield, PPL	**37a**	93 % ee, 43 % yield	**37b** [24]
n-C_7H_{15}	85 % ee, 43 % yield, PPL	**38a**	85 % ee, 45 % yield	**38b** [24]
n-C_8H_{17}	83 % ee, 48 % yield, PPL	**39a**	88 % ee, 5 % yield	**39b** [24]



R = Et	75 % ee, PPL	**40** [25]
R = *n*-C_7H_{15}	76 % ee, PPL	**41** [25]

Table 11.1-13. (cont.).

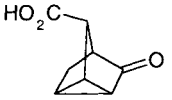
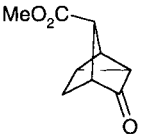
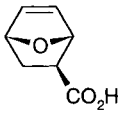
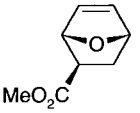
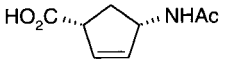
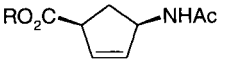
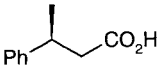
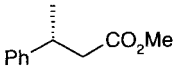
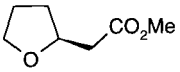
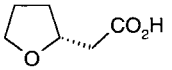
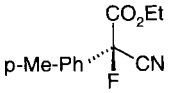
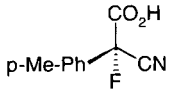
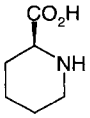
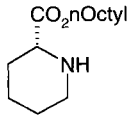
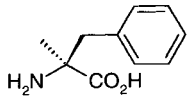
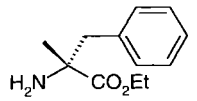
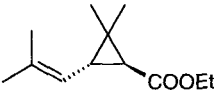
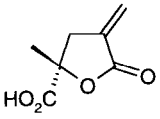
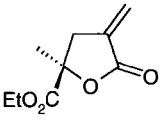
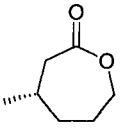
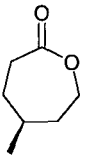
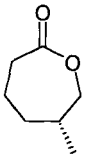
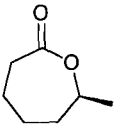
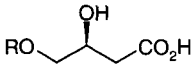
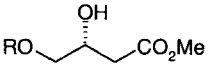
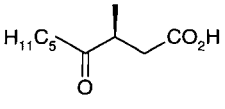
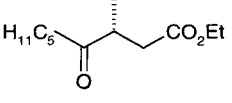
 >99 % ee, 30 % yield, CAL-A >85 % ee, 54 % yield, CAL-A	**42a** [26] **42a** [27]	 60 % ee, 56 % yield >99 % ee, 41 % yield	**42a** [26] **42a** [27]
 –, 60 % conversion, CRL	**43a** [28]	 92 % ee, –	**43b** [28]
 >99 % ee, –, CCL	**44a** [29]	 R *n*-Bu >99 % ee, 42 % yield *n*-Hex >99 % ee, 44 % yield	**44b** [29]
 89 % ee, –, PCL	**45a** [30]	 >98 % ee, 46 % yield, PCL	**45b** [30]
 84 % ee, 80 % yield, CCL	**46a** [31]	 –, 97 % yield	**46b** [31]
 99 % ee, 41 % yield, CRL	**47a** [32]	 98 % ee, 46 % yield	**47b** [32]
 93 % ee, 19 % yield, ANL (purified)	**48a** [33]	 73 % ee, –	**48b** [33]
 96 % ee, 40 % yield, lipase L	**49a** [34]	 >99 % ee, 50 % yield	**49b** [34]

Table 11.1-13. (cont.).

	50a [35]		**50b** [35]
96 % ee, 42 % yield, CCL		91 % ee, 42 % yield	
	**51** [36]		**51b** [36]
89 % ee, –, PPL, 28 % conversion 82 % ee. –; CRL, 21 % conversion		35 % ee, – 22 % ee, –	
	**52** [37]		**53** [37]
82 % ee, CAL, 47 % conversion		72 % ee, CAL, 59 % conversion	
	**54** [37]		**55** [37]
92 % ee, CAL, 50 % conversion		94 % ee, CAL, 50 % conversion	
			
R			
4-MeO-C_6H_4 99 % ee, –, PCL	**56a**	97 % ee, –, PCL	**56b** [38]
2-Allyl-C_6H_4 98 % ee, –, PCL	**57a**	98 % ee, –, PCL	**57b** [38]
2-Naphthyl 99 % ee, –, PCL	**58a**	98 % ee, –, PCL	**58b** [38]
	**59a** [39]		**59b** [39]
98 % ee, 13 % yield, PPL 93 % ee, 13 % yield, PCL		26 % ee, 67 % yield 10 % ee, 80 % yield	

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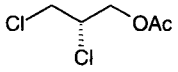
made in the case of the dihydropyridine ester **14** and ***ent*-14**. Both enantiomers are obtained with high *ee* values and in high yields by *Pseudomonas* sp. lipase-catalyzed hydrolysis merely upon changing the reaction medium from diisopropyl ether to cyclohexane, both saturated with water. The limitations of the lipase-catalyzed hydrolysis of carboxylic acid esters are evident too. Whereas the cyclohexenoid diester **10** is obtained through pig pancreas lipase-catalyzed hydrolysis with high enantioselectivity, the cyclopentanoid monoester **11** is formed only with low selectivity and the cyclopentanoid diester **12** is not a substrate for pig pancreas lipase. An interesting example for the use of a cholesterol esterase is the cyclopentanoid monoester **15**.

Monoesters of Table 11.1-12 which can be obtained with other hydrolases as such or of opposite configuration are contained in Tables 11.1-1, 11.1-2 and 11.1-7.

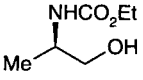
The usefulness of lipases for the enantiomer-differentiating hydrolysis of carboxylic acid esters and lactones is impressively demonstrated by examples **1–59** of Table 11.1-13. This broad substrate spectrum is covered mainly by lipases from *Candida cylindracea* (*rugosa*), pig pancreas and several *Pseudomonas* sp. lipases. Carboxylic acid esters having the alkoxycarbonyl group attached to a secondary, tertiary or even quaternary carbon atom are substrates. Thus, in contrast to

Table 11.1-14. Lipase-catalyzed enantiomer-differentiating hydrolysis of esters of racemic primary alcohols in aqueous solution (PPL pig pancreas lipase, PCL *Pseudomonas cepacia* lipase, PCL-A *Pseudomonas cepacia* lipase, Sumitomo, PSL *Pseudomonas* sp. lipase, PAL *Pseudomonas aeruginosa* lipase, HLL *Humicola lanuginosa* lipase, CAL-B *Candida antarctica* B lipase, CRL *Candida rugosa* lipase).



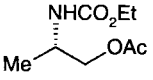
1 [1]

90 % ee, –, pancreatin
60 % conversion[a]



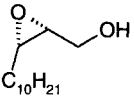
2a [2]

90 % ee, 30 % yield, pancreatin



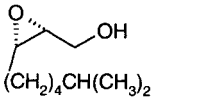
2b [2]

≥95 % ee, –



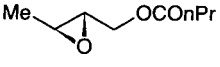
3 [3]

≥95 % ee, 32 % yield, PPL
60 % conversion[a, b]



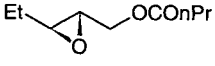
4 [3]

≥95 % ee, 20 % yield, PPL
20 % conversion[a, b]



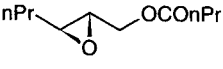
5 [4]

≥95 % ee, –, PPL
60 % conversion[a]



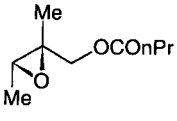
6 [4]

≥95 % ee, –, PPL
58 % conversion[a]



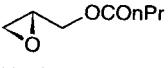
7 [4]

56 % ee, –, PPL
60 % conversion[a]



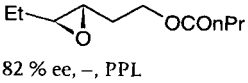
8 [4]

73 % ee, –, PPL
60 % conversion[a]



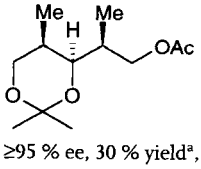
9 [4]

77 % ee, –, PPL
60 % conversion[a]



10 [4]

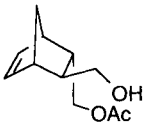
82 % ee, –, PPL
60 % conversion[a]



11 [5]

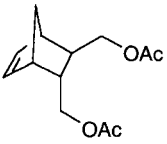
≥95 % ee, 30 % yield[a], PPL

Table 11.1-14. (cont.).



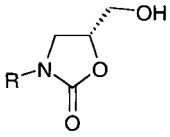
12a [6]

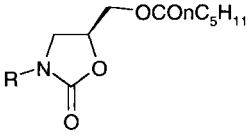
91 % ee, 45 % yield, PSL



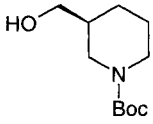
12b [6]

≥95 % ee, 47 % yield



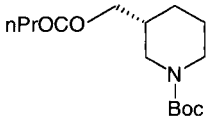


R = H	97 % ee, –, PAL	**13a**	99 % ee, –	**13b** [7]
R= *i*-Pr	≥97 % ee, –, PAL	**14a**	≥97 % ee, –	**14b** [8, 9]
R = *t*-Bu	≥97 % ee, –, PAL	**15a**	≥97 % ee, –	**15b** [8, 9]
R = Ph	96 % ee, –, PAL	**16a**	100 % ee, –	**16b** [7]



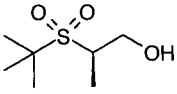
17a [10]

94 % ee, –, PCL
53 % conversion



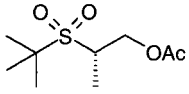
17b [10]

91 % ee, –
42 % conversion



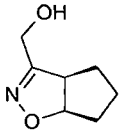
18a [11]

61 % ee, 54 % yield, CAL-B



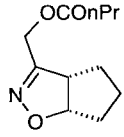
18b [11]

99 % ee, 37 % yield



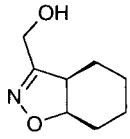
19a [12]

87 % ee, –, PCL, 46 % conversion



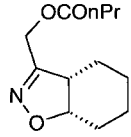
19b [12]

>98 % ee, –, PCL, 61 % conversion



20a [12]

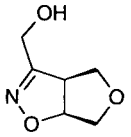
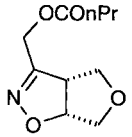
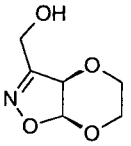
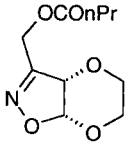
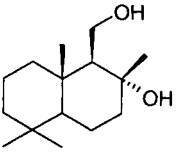
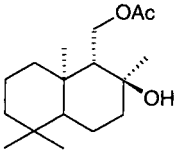
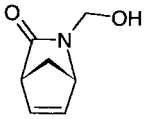
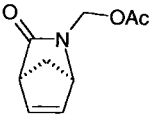
87 % ee, –, PCL, 47 % conversion

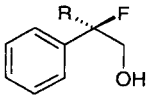


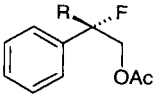
20b [12]

>98 % ee, –, PCL, 60 % conversion

Table 11.1-14. (cont.).

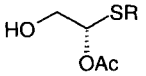
 90 % ee, –, PCL, 50 % conversion	**21a** [12]	 90 % ee, –, PCL, 50 % conversion	**21b** [12]
 93 % ee, –, PCL, 46 % conversion	**22a** [12]	 >98 % ee, –, PCL, 55 % conversion	**22b** [12]
 >99 % ee, 11 % yield, PCL	**23a** [13]	 16 % ee, 85 % yield	**23b** [13]
 95 % ee, 41 % yield, PCL	**24a** [14]	 96 % ee, 41 % yield, PCL	**24b** [14]





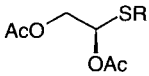
	R			R	
91 % ee, 35 % yield, PCL	Me	**25a** [15]	>98 % ee, 27 % yield	Me	**25b** [15]
86 % ee, 29 % yield, PCL	*n*-Pr	**26a** [15]	92 % ee, 34 % yield	*n*-Pr	**26b** [15]
88 % ee, 31 % yield, PCL	*n*-Bu	**27a** [15]	96 % ee, 33 % yield	*n*-Bu	**27b** [15]

Table 11.1-14. (cont.).



28a [16]

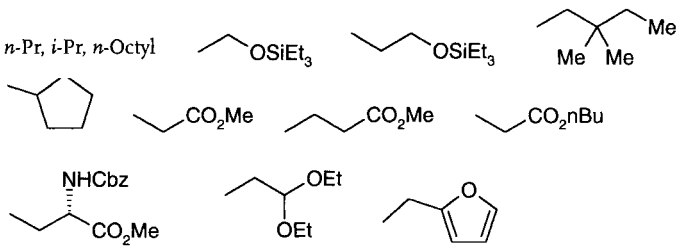
48–>96 % ee, 34–50 % yield, PCL

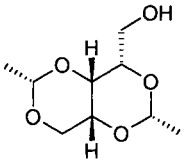


28b [16]

47–>89 % ee, 34–57 % yield, PCL

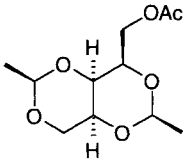
R





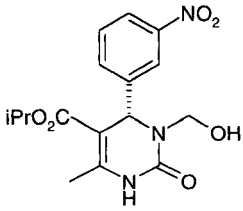
29a [17]

>99 % ee, 43 % yield, PPL



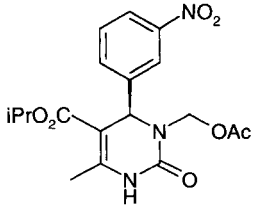
29b [17]

>99 % ee, 50 % yield, PPL



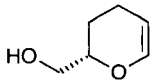
30a [18]

96 % ee, 50 % yield, HLL



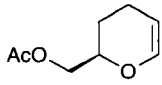
30b [18]

98 % ee, 45 % yield



31a [19]

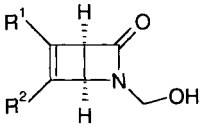
91 % ee, 26 % yield, PPL

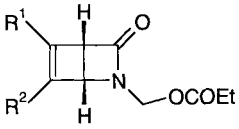


31b [19]

37 % ee, 72 % yield, PPL

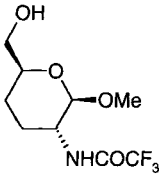
Table 11.1-14. (cont.).



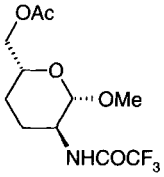


	R^1	R^2		lipase		
96 % ee, 23 % yield	H	CO_2Me	**32a** [20]	PCL	33 % ee, 72 % yield	**32b** [20]
>98 % ee, 21 % yield				PSL	44 % ee, 72 % yield	
>98 % ee, 34 % yield	H	H	**33a** [20]	PCL	51 % ee, 57 % yield	**33b** [20]
>98 % ee, 25 % yield				PSL	34 % ee, 73 % yield	
95 % ee, 28 % yield	OMe	H	**34a** [20]	PCL	49 % ee, 65 % yield	**34b** [20]
97 % ee, 27 % yield				PSL	40 % ee, 67 % yield	
92 % ee, 12 % yield	R^1 =		**35a** [20]	PCL	18 % ee, 72 % yield	**35b** [20]
92 % ee, 28 % yield				PSL	52 % ee, 61 % yield	

R^2 = H

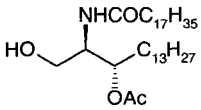


36a [21]

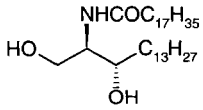


36b [21]

>98 % ee, 37 % yield, PPL, 38 % conversion	–,–
–,–, PPL, 70 % conversion	>98 % ee, 29 % yield
79 % ee, –, PSL, 36 % conversion	–,–
–,–, PSL, 69 % conversion	>98 % ee



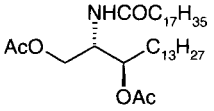
37a [22]



37b [22]

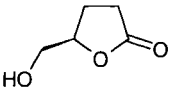
49 % ee, 54 % yield, PCL-A	98 % ee, 7 % yield
96 % ee, 41 % yield, PCL-A (immobilized)	

Table 11.1-14. (cont.).



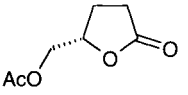
37c [22]

87 % ee, 38 % yield, PCL-A
69 % ee, 58 % yield, PCL-A (immobilized)



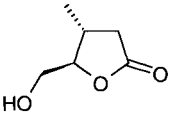
38a [23]

79 % ee, –, PCL, 50 % conversion



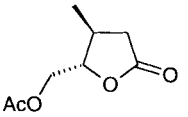
38b [23]

84 % ee, –



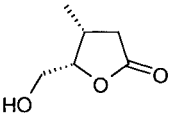
39a [23]

89 % ee, –, PCL, 53 % conversion



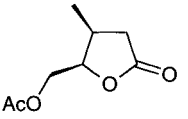
39b [23]

94 % ee, –



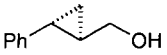
40a [24]

96 % ee, –, PCL, 50 % conversion



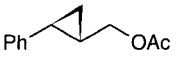
40b [23]

95 % ee, –



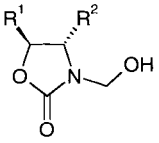
41a [25]

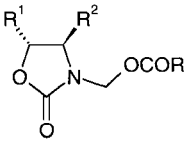
78 % ee,–, PCL
82 % ee, –, CAL-B



41b [24]

53 % ee, –
78 % ee, –

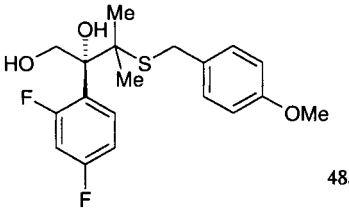




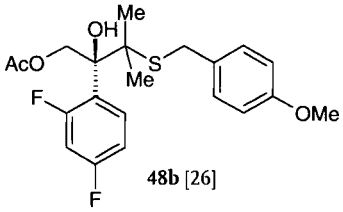
	R	R^1	R^2		lipase		
75 % ee, 42 % yield	Et	Ph	H	**42a** [25]	PCL	70 % ee, 46 % yield,	**42b** [25]
62 % ee, 50 % yield	*t*-Bu	Ph	H	**42a** [25]	PCL	67 % ee, 47 % yield	**43b** [25]
89 % ee, 51 % yield	Et	H	Ph	**44a** [25]	PCL	93 % ee, 42 % yield	**44b** [25]

Table 11.1-14. (cont.).

87 % ee, 42 % yield	Et	H	Ph	**44a**	PSL	90 % ee, 46 % yield	**44b** [25]
97 % ee, 44 % yield	*n*-Pent	H	Ph	**44a**	PCL	92 % ee, 50 % yield	**45b** [25]
94 % ee, 43 % yield	Et	H	Bn	**46a**	PCL	91 % ee, 46 % yield	**46b** [25]
72 % ee, 46 % yield	Et	H	Bn	**46a**	PSL	76 % ee, 44 % yield	**46b** [25]
69 % ee, 52 % yield	Et	H	Et	**47a**	PCL	98 % ee, 40 % yield	**47b** [25]
97 % ee, 50 % yield, 0 °C	Et	H	Et	**47a**	PCL	87 % ee, 47 % yield	**47b** [25]



48a [26]



48b [26]

89 % ee, –, CRL	87 % ee, –
90 % ee, –, lipase MY	77 % ee, –
96 % ee, 54 % yield, lipase OF-360	84 % ee, 59 % yield
91 % ee, –, CCL	80 % ee, –

a The other product (alcohol or ester) was not isolated. **b** Acetate was hydrolyzed.

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uncatalyzed ester hydrolysis, steric hindrance, at least for the known examples **14–17** and **26–30** in the enzyme-catalyzed hydrolysis, poses no problem. In substrates containing two alkoxycarbonyl groups, one attached to a secondary carbon and the other one to a tertiary carbon, the former is hydrolyzed more readily, as shown for **3–5**. Esters with the alkoxycarbonyl group attached to quaternary carbon are readily hydrolyzed, as demonstrated for **17**, **26–30**, **47**, **49**, **51** (Table 11.1-13).

Group selectivity is also observed between an ester group and a thioester group or an ester and a lactone moiety, as exemplified by **12** and **51**, respectively. Acyclic as well as cyclic carboxylic acid esters are substrates for enantiomer-selective hydrolysis catalyzed by lipases. High enantioselectivities are observed not only for those esters having a chiral center in α-position but also for those having the chiral center in β-position. A spectacular example in this regard is the acetoxy-substituted carboxylic acid **33**, where the chiral center is separated by eight methylene groups from the carboxyl group. This acid is obtained by a *Candida cylindracea* lipase-catalyzed hydrolysis of the corresponding racemic butyl ester with very high enantioselectivity. Surprisingly, the hydrolysis of the corresponding methyl ester proceeds with a much lower enantioselectivity. Lipase-catalyzed enantiomer-differentiating hydrolysis has been utilized with much success for the synthesis of α-hydroxy and α-acetoxy carboxylic acids (**6**, **7**, **24** and **25**). A series of vinylogous α-hydroxy carboxylic acids **34–39** is also accessible.

The two α-amino acids **48** and **49** with unprotected amino groups are hydrolyzed with high enantioselectivity. The series of methyl-substituted seven-membered lactones **52–55** (Table 11.1-13) are converted in the presence of *Candida antarctica* lipase yielding the slow-reacting lactones with *ee* values between 72 and 94%.

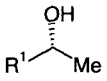
Acids, monoesters and lactones of Table 11.1-13 which can be obtained with other hydrolases as such or of opposite configuration are contained in Table 11.1-5.

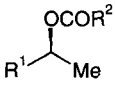
Lipase-catalyzed enantiomer-differentiating hydrolysis of acylated racemic primary alcohols covers a broad range of substrates (**1–48**) summarized in Table 11.1-14, including epoxy alcohols (**3–10**), amino alcohols (**2**, **17**, **36**, **37**) and acylated γ-hydroxymethyl γ-lactones (**38–40**). By means of incorporating the amino and the secondary hydroxyl group into a heterocyclic ring system, selectively protected amino diols are accessible by *Pseudomonas aeruginosa* lipase-catalyzed hydrolysis (**13–16**). 3-Hydroxymethyl-D^2-isoxazoline butyrates **19–22** (Table 11.1-14) have been resolved with high selectivity in the presence of *Pseudomonas cepacia* lipase.

Monoacetates and alcohols of Table 11.1-14 which can be obtained with other hydrolases as such or of opposite configuration are contained in Table 11.1-19.

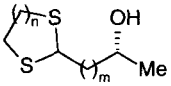
Given the experimental simplicity and the potential scale of reaction, lipase-catalyzed enantiomer-differentiating hydrolysis of racemic acylated secondary alcohols is today one of the best methods for the synthesis of optically active secondary alcohols. From the list of the tabulated examples **1–170** (Table 11.1-15) one gets the impression that there is almost no restriction in regard to the substrate structure. Because of the number of lipases available either as isolated enzymes or contained in the various organisms, it seems possible to find the right lipase for almost every substrate. Highly enantiomer-selective hydrolysis and alcoholysis of esters of a wide structural range of secondary alcohols by the different lipases are possible. Not only

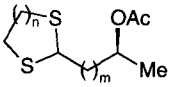
Table 11.1-15. Lipase-catalyzed enantiomer-differentiating hydrolysis of esters of racemic acyclic secondary alcohols in aqueous solution (CCL *Candida cylindracea* lipase, PSL *Pseudomonas* sp. lipase, PFL *Pseudomonas fluorescens* lipase, PAL *Pseudomonas aeruginosa* lipase, ASL *Alcaligenes* sp. lipase, ANL *Aspergillus niger* lipase, PCL *Pseudomonas cepacia* lipase, ROL *Rhizopus oryzae* lipase, MML *Mucor miehei* lipase, CAL-B *Candida antarctica* B lipase, LIP *Pseudomonas* sp. lipase – Toyobo, HSL *Humicola* sp. lipase).



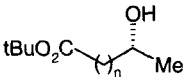


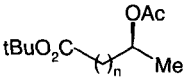
		R^1	R^2	lipase		
1a	90 % ee, 39 % yield	Et	*n*-Pr	CCl	88 % ee, 40 % yield	**1b** [1]
2a	≥99 % ee, 48 % yield	Ph	Me	PSL	≥99 % ee, 48 % yield	**2b** [2]
3a	97 % ee, 47 % yield	4-Me-C_6H_4	Me	PSL	99 % ee, 45 % yield	**3b** [2]
4a	80 % ee, 46 % yield	4-MeO-C_6H_4	Me	PSL	80 % ee, 47 % yield	**4b** [2]
5a	95 % ee, 47 % yield	$PhCH_2$	Me	PSL	97 % ee, 48 % yield	**5b** [2]
6a	95 % ee, 46 % yield	4-Pyridyl	Me	PSL	89 % ee, 47 % yield	**6b** [2]
7a	≥99 % ee, 43 % yield	2-Naphthyl	Me	PSL	≥99 % ee, 46 % yield	**7b** [2]
8a	95 % ee, 50 % yield	Ph	CH_2Cl	PSL	96 % ee, 44 % yield	**8b** [2]



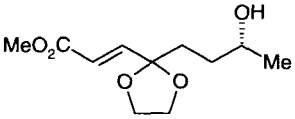


m = 0, n = 1	99 % ee, 37 % yield, PFL 46 % conversion	**9a**	95 % ee, 40 % yield	**9b** [3]
			95 % ee, 40 % yield	**10b** [3]
m = 0, n = 2	99 % ee, 35 % yield, PFL 46 % conversion	**10a**		
			95 % ee, 40 % yield	**11b** [3]
m = 1, n = 1	95 % ee, 36 % yield, PFL 48 % conversion	**11a**		
			95 % ee, 40 % yield	**12b** [3]
m = 1, n = 2	99 % ee, 35 % yield, PFL 48 % conversion	**12a**		

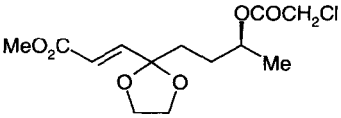




n = 0	≥99 % ee, –, PSL	**13a** [4a]	96 % ee, –	**13b** [4]
n = 1	≥99 % ee, –, PSL	**14** [4]	–	
n = 5	≥99 % ee, –, PSL	**15** [4]	–	
n = 10	≥99 % ee, –, PSL	**16** [4]	–	

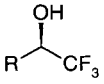


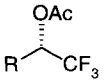
93 % ee, –, PSL **17a** [5]



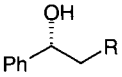
≥99 % ee, – **17b** [5]

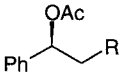
Table 11.1-15. (cont.).



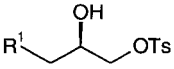


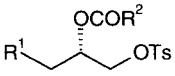
R				
Ph	57 % ee, –, CCL	**18a** [6]	–, –	**19b** [6]
CH_2Ph	94 % ee, –, CCL	**19a** [6]	98 % ee, –	
$(CH_2)_2Ph$	98 % ee, –, CCL	**20a** [6]	–, –	
2-styryl	93 % ee, –, CCL	**21a** [6]	–, –	
CH_2CO_2Et	96 % ee, –, CCL	**22a** [6]	–, –	
CH_2CO_2Hex	90 % ee, –, CCL	**23a** [6]	–, –	
	28–50 % conversion		–, –	



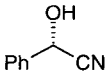


R				
Cl	100 % ee, 24 % yield, PSL	**24a**	100 % ee, 29 % yield	**24b** [7]
Br	94 % ee, 24 % yield, PSL	**25a**	100 % ee, 11 % yield	**25b** [7]
	50 % conversion			
n-C_5H_{11}	92 % ee, –, PSL	**26a**	≥99 % ee, –	**26b** [8]
n-C_6H_{13}	98 % ee, –, PSL	**27a**	≥99 % ee, –	**27b** [8]
n-C_7H_{15}	97 % ee, –, PSL	**28a**	98 % ee, –	**28b** [8]
	42–53 % conversion			



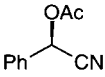


R^1			R^2	
Me	≥99 % ee, 40 % yield, PAL	**29a**	Me, Pr≥99 % ee, 35 % yield	**29b** [9]
Et	≥99 % ee, 46 % yield, PAL	**30a**	Me, Pr≥99 % ee, 44 % yield	**30b** [9]
CH_2Cl	≥99 % ee, 46 % yield, PAL	**31a**	Me, Pr≥99 % ee, 45 % yield	**31b** [10]



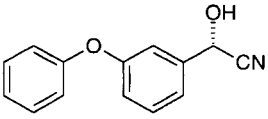
32a [11]

–, –



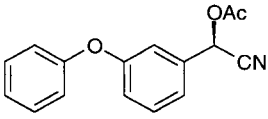
32b [11]

≥98 % ee, 42 % yield, PSL



33a [11, 12]

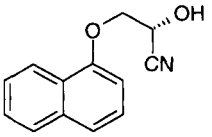
98 % ee, –



33b [11, 12]

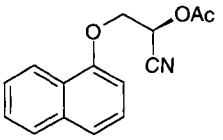
98 % ee, 40 % yield, ASL
(pH 4–5)
87 % ee, –, PSL

Table 11.1-15. (cont.).



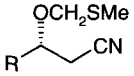
34a [13]

–

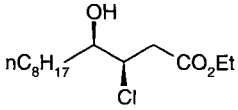


34b [13]

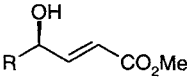
87 % ee, 39 % yield, PSL



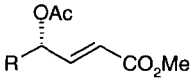
R		
Me	≥98 % ee, –, PFL	35 [14]
Ph	≥98 % ee, –, PFL 60–64 % conversion	36 [14]
$Ph(CH_2)_2$	≥98 % ee, –, PFL	37 [14]
2-styryl	≥98 % ee, –, PFL	38 [14]



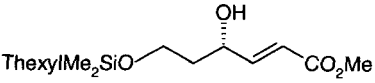
94 % ee, 31 % yield, PFL 39 [15]



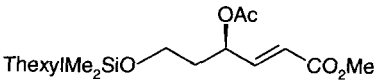
R = Me ≥95 % ee, 37 % yield, PFL 40a [16]
R = Et ≥95 % ee, 44 % yield, PFL 41a [16]



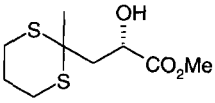
91 % ee, 39 % yield 40b [16]
≥95 % ee, 45 % yield 41b [16]



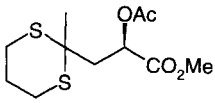
72 % ee, 57 % yield, PFL 42a [16]



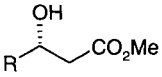
≥95 % ee, 35 % yield 42b [16]



97 % ee, 41 % yield, PFL 43a [17]



96 % ee, 26 % yield 43b [17]



R				
Et	74 % ee, –, CCL	44a	42 % ee, –	44b [18]
$C_{11}H_{23}$	84 % ee, –, CCL	45a	75 % ee, –	45b [18]
$(CH_2)_4CH(C_4H_9)_2$	92 % ee, –, CCL 40 % conversion	46a	50 % ee, – 60 % conversion	46b [18]

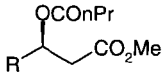
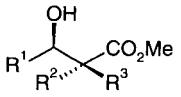
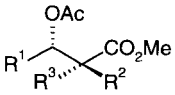
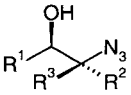


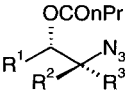
Table 11.1-15. (cont.).



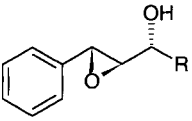


		R^1	R^2	R^3		
47a	66 % ee, 56 % yield, ANL	2-furyl	H	Me	≥99 % ee, 33 % yield	**47b** [19]
48a	67 % ee, 64 % yield, ANL	2-thiophenyl	H	Me	91 % ee, 35 % yield	**48b** [19]
49a	64 % ee, 51 % yield, ANL	2-(2-butenyl)	H	Me	≥99 % ee, 38 % yield	**49b** [19]
50a	75 % ee, 51 % yield, ANL	2-furyl	Me	H	98 % ee, 32 % yield	**50b** [19]
51a	85 % ee, 53 % yield, ANL	2-thiophenyl	Me	H	≥99 % ee, 47 % yield	**51b** [19]
52a	79 % ee, 51 % yield, ANL	2-(2-butenyl)	Me	H	≥99 % ee, 44 % yield	**52b** [19]



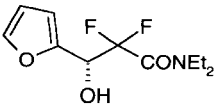


		R^1	R^2	R^3	lipase		
	–	*t*-Bu	H	H	CCL	≥98 % ee, –, ANL	**53b** [20]
	–	Ph	H	H	PFL	≥98 % ee, –, ANL	**54b** [20]
	–	Et	Et	H	PFL	≥98 % ee, –, ANL	**55b** [20]
56a [21]	≥98 % ee, –	Et	H	Et	PFL		
57a [21]	≥98 % ee, – 30–40 % conversion	*n*-Pr	H	*n*-Pr	PFL	52–60 % conversion	



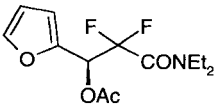
other product not isolated

R = Et	100 % ee, 50 % yield, PPL	**58a** [21]
R = *n*-Pr	60 % ee, 50 % yield, PPL	**59a** [21]
R = $(CH_2)_2CO_2Et$	56 % ee, 22 % yield, PPL	**60a** [21]



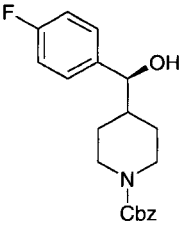
61a [22]

58 % ee, –, CCL



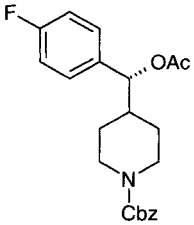
61b [22]

96 % ee, –



62a [23]

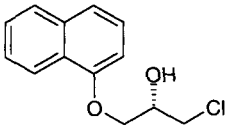
>97 % ee, 58 % yield, CCL



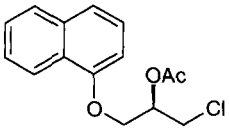
62b [23]

≥97 % ee, 42 % yield

Table 11.1-15. (cont.).



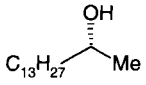
63a [24]

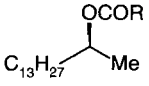


63b [24]

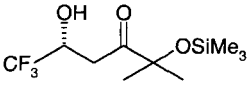
≥97 % ee, 41 % yield, PCL

88 % ee, 43 % yield

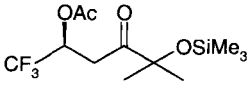




52 % ee, 40 % yield, PCL	**64a**	R = Ac	54 % ee, 41 % yield	**64b** [25]
95 % ee, 23 % yield, PCL	**65a**	R = OCH_2CCl_3	35 % ee, 42 % yield	**65b** [25]



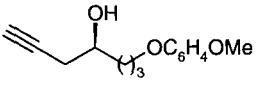
66a [26]



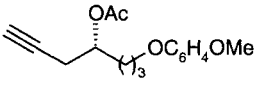
66b [26]

≥95 % ee, 27 % yield, PCL

–, 48 % yield
(hydrolysis with PFL gives the (*S*)-alcohol with ≥95 % ee)



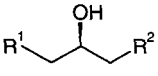
67a [27]

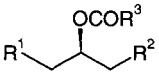


67b [27]

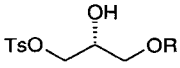
98 % ee, 53 % yield, PCL
(buffer : acetone = 9 : 1)

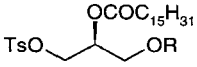
≥99 % ee, 42 % yield





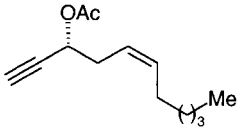
	R^1	R^2	R^3			
≥98 % ee, –, PCL	H	O-*t*-Bu	CH_2Cl	**68a**	≥98 % ee, –	**68b** [28, 29]
≥98 % ee, –, PCL	Me	O-*t*-Bu	CH_2Cl	**69a**	≥98 % ee, –	**69b** [28, 29]
88 % ee, –, PCL	H	SPh	Me	**70a**	90 % ee, –,	**70b** [28, 29]
≥98 % ee, –, PCL	Me	SPh	Me	**71a**	91 % ee, –	**71b** [28, 29]
≥96 % ee, –, PCL	H	SPh	CH_2Cl	**72a**	≥96 % ee, –	**72b** [28, 29]
≥96 % ee, –, PCL	Me	S-*t*-Bu	CH_2Cl	**73a**	≥96 % ee, –	**73b** [28, 29]
≥96 % ee, –, PCL	*n*-Pr	S-*t*-Bu	CH_2Cl	**74a**	≥96 % ee, –	**74b** [28, 29]
≥96 % ee, –, PCL	*n*-Pent	S-*t*-Bu	CH_2Cl	**75a**	≥96 % ee, –	**75b** [28, 29]
≥96 % ee, –, PCL	*n*-Non	S-*t*-Bu	CH_2Cl	**76a**	≥96 % ee, –	**76b** [28, 29]





	R			
99 % ee, 44 % yield, PCL	$C_{16}H_{31}$	**77a**	≥95 % ee, 46 % yield	**77b** [30]
99 % ee, 47 % yield, PCL	$C_{10}H_{21}$	**78a**	≥95 % ee, 42 % yield	**78b** [30]
99 % ee, 45 % yield, PCL	C_4H_9	**79a**	≥95 % ee, 43 % yield	**79b** [30]

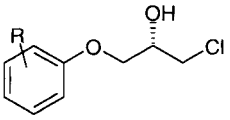
Table 11.1-15. (cont.).



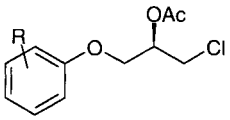
80 [31]

96 % ee, 27 % yield, PPL

other product not isolated



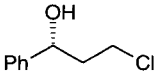
81a [32]



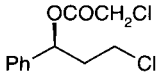
81b [32]

(R = H, Me, OMe, NO_2, allyl, O-allyl, c-C_5H_{11}
39–99 % ee, 31–54 % yield, PSL

67–99 % ee, 31–52 % yield



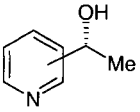
82a [33]



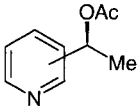
82b [33]

97 % ee, 50 % yield, PSL

≥99 % ee, 50 % yield



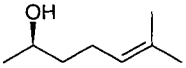
83a [34]



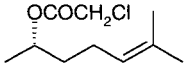
83b [34]

(2–4)
≥95 % ee, 37–55 % yield, PSL

≥95 % ee, 37–45 % yield



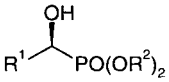
84a [35]

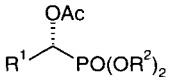


84b [35]

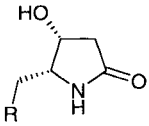
89 % ee, 53 % yield, PSL

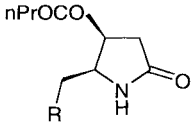
100 % ee, 48 % yield





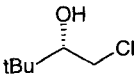
	R^1	R^2			
≥99 % ee, 31 % yield, ROL	Ph	Me	**85a**	90 % ee, 35 % yield	**85b** [36]
≥99 % ee, 37 % yield, ROL	Ph	*i*-Pr	**86a**	86 % ee, 46 % yield	**86b** [36]
50 % ee, 21 % yield, ROL	Me	Me	**87a**	9 % ee, 29 % yield	**87b** [36]





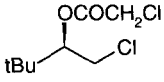
	R			
≥99 % ee, –, CCL	*i*-Pr	**88a** [37]	≥99 % ee, 43 % yield	**88b** [37]
≥99 % ee, –, CCL	Ph	**89a** [37]	≥99 % ee, 43 % yield	**89b** [37]

Table 11.1-15. (cont.).



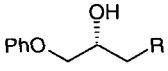
90a [38]

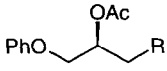
91 % ee, 38 % yield, PSL



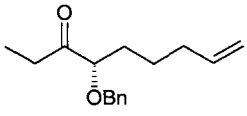
90b [38]

≥98 % ee, 42 % yield



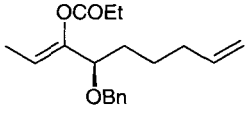


	R			
92 % ee, 31 % yield, PSL	Cl	**91a**	97 % ee, 41 % yield	**91b** [39]
86 % ee, 46 % yield, PSL	Br	**92a**	97 % ee, 41 % yield	**92b** [39]
91 % ee, 48 % yield, PSL	N_3	**93a**	84 % ee, 48 % yield	**93b** [39]



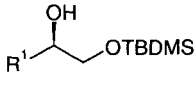
94a [40]

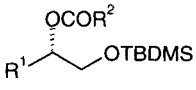
32 % ee, 66 % yield, CCL (isolated as alcohol obtained with $NaBH_4$)



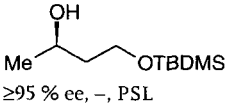
94b [40]

≥99 % ee, 22 % yield



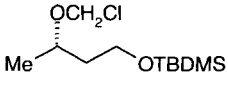


	R^1	R^2			
≥95 % ee, –, PSL	Me	*n*-Pr	**95a**	≥95 % ee, –	**95b** [41]
≥95 % ee, –, PSL	Et	*n*-Pr	**96a**	≥95 % ee, –	**96b** [41]
≥95 % ee, –, PSL	CH_2Cl	*n*-Pr	**97a**	≥95 % ee, –	**97b** [41]
≥95 % ee, –, PSL	$CH{=}CH_2$	$ClCH_2$	**98a**	91 % ee, –	**98b** [41]
≥95 % ee, –, PSL	$CH_2OCH{=}CH_2$	$ClCH_2$	**99a**	51 % ee, –	**99b** [41]



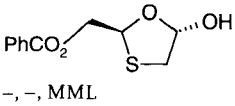
100a [41]

≥95 % ee, –, PSL



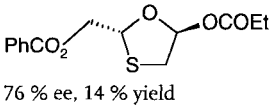
100b [41]

≥95 % ee, –



101a [42]

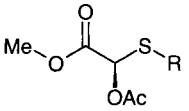
–, –, MML

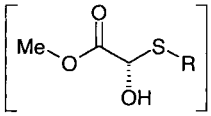


101b [42]

76 % ee, 14 % yield

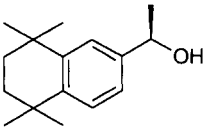
Table 11.1-15. (cont.).





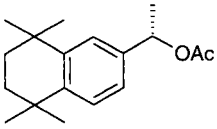
could not be isolated

R		
-$CH_2CH(OMe)_2$	>95 % ee, 45 % yield, PFL	**102** [43]
-$CH_2CH(OEt)_2$	>95 % ee, 49 % yield, PFL	**103** [43]
-$CH_2CH(OBn)_2$	65 % ee, 48 % yield, PFL	**104** [43]
-$(CH_2)_2CH(OMe)_2$	>95 % ee, 45 % yield, PFL	**105** [43]
n-Bu	>95 % ee, 47 % yield, PFL	**106** [43]
-$(CH_2)_2OSiEt_3$	81 % ee, 47 % yield, PFL	**107** [43]
-$(CH_2)_3OSiEt_3$	85 % ee, 48 % yield, PFL	**108** [43]



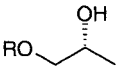
109a [44]

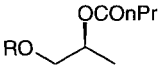
>95 % ee, 44 % yield, PCL



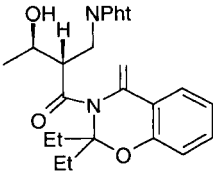
109b [44]

>95 % ee, 42 % yield, PCL



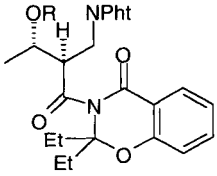


R				
Ph	98 % ee, –, PCL	**110a**	99 % ee, –, PCL	**110b** [45]
Ph	99 % ee, –, CAL-B		99 % ee, –, CAL-B	
Bn	95 % ee, –, PCL	**111a**	97 % ee, –, PCL	**111b** [45]
Bn	97 % ee, –, CAL-B		97 % ee, –, CAL-B	
-$(CH_2)_2Ph$	98 % ee, –, CAL-B	**112a**	97 % ee, –, CAL-B	**112b** [45]



113a [46]

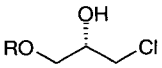
>99 % ee, 49 % yield, ASL

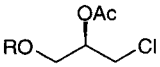


R = $CO(CH_2)_2Me$

113b [46]

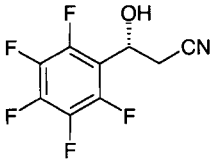
>99 % ee, 50 % yield





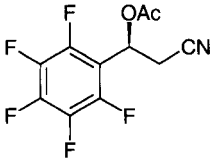
R				
2-Naphthyl	95 % ee, –, CAL-B	**114a**	95 % ee, 47 % yield	**114b** [47]
4-AcNH-C_6H_4	95 % ee, –, CAL-B	**115a**	79 % ee, 40 % yield	**115b** [47]
4-[MeO-$(CH_2)_2$]-C_6H_4 C_6H_4	95 % ee, –, CAL-B	**116a**	70 % ee, 37 % yield	**116b** [47]

Table 11.1-15. (cont.).



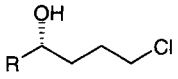
117a [48]

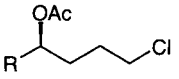
93 % ee, 40 % yield, PFL
>98 % ee, 41 % yield, LIP



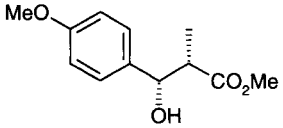
117b [48]

82 % ee, 37 % yield, PFL
>99 % ee, 521 % yield, LIP



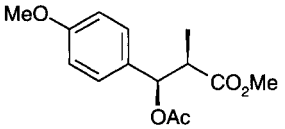


R					
Ph	89 % ee, –, PCL	**118a**	80 % ee, –,	**118b** [49]
4-F-C_6H_4	93 % ee, –, PCL	**119a**	78 % ee, –,	**119b** [49]
4-*t*-Bu-C_6H_4	>95 % ee, –, PCL	**120a**	>95 % ee, –,	**120b** [49]



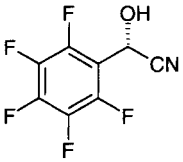
121a [50]

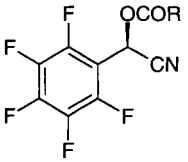
94 % ee, 51 % yield, ANL



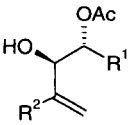
121b [50]

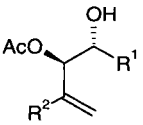
>99 % ee, 48 % yield

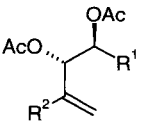




R				
Me	94 % ee, 39 % yield, LIP	**122a**	85 % ee, 39 % yield	**122b** [51]
Et	97 % ee, 38 % yield, LIP	**122a**	92 % ee, 46 % yield	**123b** [51]
Ph	89 % ee, 32 % yield, LIP	**122a**	96 % ee, 43 % yield	**124b** [51]



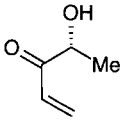




not separated

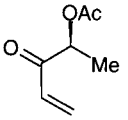
R^1	R^2					
Me	H	96 % ee, CAL-B	97 % ee	**125a,b**	>98 % ee, 42 % yield	**125c** [52]
Et	H	>98 % ee, CAL-B	93 % ee	**126a,b**	>98 % ee, 37 % yield	**126c** [52]
i-Pr	H	>98 % ee, CAL-B	>98 % ee	**127a,b**	63 % ee, 39 % yield	**127c** [52]
Me	Me	>98 % ee, CAL-B	>96 % ee	**128a,b**	96 % ee, 38 % yield	**128c** [52]

Table 11.1-15. (cont.).



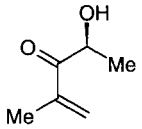
129a [53]

85 % ee, –, CAL-B
52 % conversion



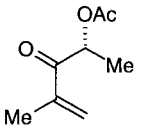
129b [53]

93 % ee, -



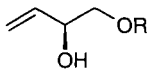
130a [53]

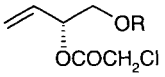
96 % ee, –, CAL-B
49 % conversion



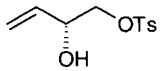
130b [53]

92 % ee, –



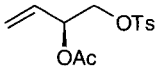


R				
$SiMe_2Thex$	97 % ee, –, PCL	**131a**	99 % ee, –	**131b** [54]
	94 % ee, –, CAL-B		95 % ee, –	
Bz	94 % ee, –, HSL	**132a**	97 % ee, –	**132b** [54]



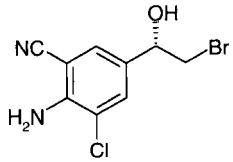
133a [54]

99 % ee, –, PCL



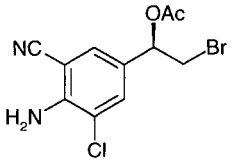
133b [54]

99 % ee, –, PCL



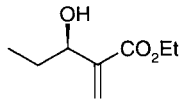
134a [55]

99 % ee, 42 % yield, CAL-B



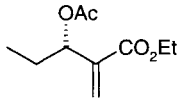
134a [55]

96 % ee, 42 % yield



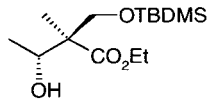
135a [56]

>99 % ee, 19 % yield, PSL



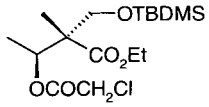
135b [56]

46 % ee, 33 % yield



136a [57]

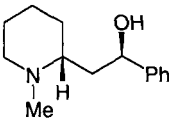
>99 % ee, 43 % yield, PCL



136b [57]

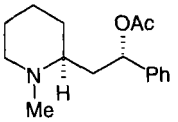
90 % ee, 46 % yield

Table 11.1-15. (cont.).



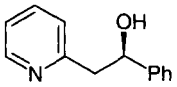
87 % ee, 39 % yield, PPL

137a [58]



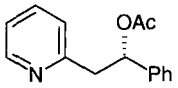
80 % ee, 45 % yield

137b [58]



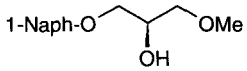
>96 % ee, 40 % yield, PPL

138a [58]



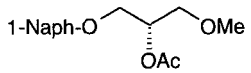
70 % ee, 48 % yield

138b [58]



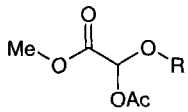
82 % ee, 47 % yield, CAL-B

139a [58]

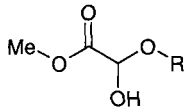


90 % ee, 38 % yield

139b [59]

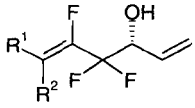


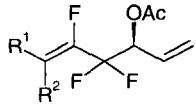
abs. config. unknown



racemic

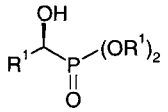
R		
$-(CH_2)_7CH_3$	>95 % ee, 43 % yield, PFL	**140** [60]
$-CH_2CH{=}CH_2$	>95 % ee, 32 % yield, PFL	**141** [60]
$-(CH_2)_2CH{=}CH_2$	90 % ee, 37 % yield, PFL	**142** [60]
$-CH_2CH{=}CHPh$	>95 % ee, 39 % yield, PFL	**143** [60]
$-CH_2Ph$	>95 % ee, 35 % yield, PFL	**144** [60]
$-(CH_2)CO_2Et$	>95 % ee, 27 % yield, PFL	**145** [60]
$-CH_2CH(OEt)_2$	>95 % ee, 37 % yield, PFL	**146** [60]



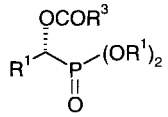


R^1	R^2				
Et	Et	>99 % ee, 38 % yield, PCL	**146a**	94 % ee, 42 % yield	**146b** [61]
		>99 % ee, 37 % yield, CAL	**147a**	59 % ee, 57 % yield	**147b** [61]
		>99 % ee, 22 % yield, CRL	**148a**	39 % ee, 48 % yield	**148b** [61]
		>99 % ee, 36 % yield, ASL	**149a**	81 % ee, 40 % yield	**149b** [61]
Me	Me	>99 % ee, 36 % yield, PCL	**150a**	91 % ee, 54 % yield	**150b** [61]
		>99 % ee, 23 % yield, CAL	**151a**	73 % ee, 21 % yield	**151b** [61]

Table 11.1-15. (cont.).



152a [62]

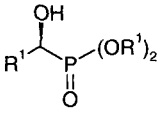


152b [62]

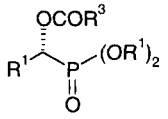
R^1 = aromatic, heteroaromatic, R^2 = Me, Et, *i*-Pr, R^3 = Me, CH_2Cl

14–95 % ee, ANL or ROL

9–70 % ee



153a [63]



153b [63]

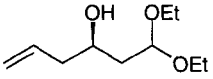
in some cases *R*

in some cases *S*

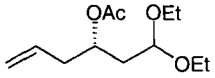
R^1 = alkyl, R^2 = Me, Et, *i*-Pr, R^3 = Me, CH_2Cl

7.5–98 % ee, ANL or ROL

3–80 % ee



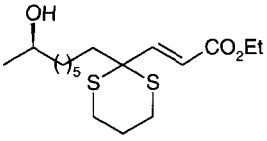
154a [64]



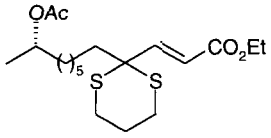
154b [64]

>98 % ee, 41 % yield, PSL

–, –



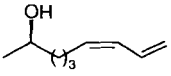
155a [65]



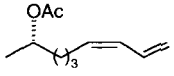
155b [65]

95 % ee, 38 % yield, PCL

–,–



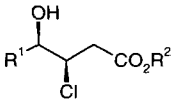
156a [66]

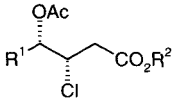


156b [66]

80 % ee, 44 % yield, PSL

87 % ee, 44 % yield





R^1	R^2				
Me	Et	>99 % ee, 29 % yield, PCL	**157a**	–, 30 % yield	**157b** [67]
Et	Me	96 % ee, 29 % yield, PCL	**158a**	–, 43 % yield	**158b** [67]
Et	Et	86 % ee, 29 % yield, PCL	**159a**	–, 22 % yield	**159b** [67]
n-C_8H_{17}	Et	98 % ee, 29 % yield, PCL	**160a**	–, 18 % yield	**160b** [67]
n-C_8H_{17}	Me	87 % ee, 35 % yield, PCL	**161a**	–, 31 % yield	**161b** [67]
n-C_8H_{17}	Et	94 % ee, 31 % yield, PCL	**162a**	–, 22 % yield	**162b** [67]

Table 11.1-15. (cont.).

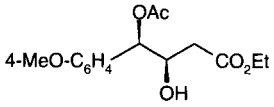
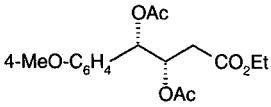
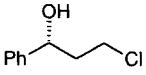
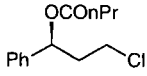
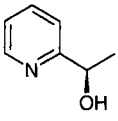
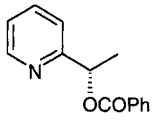
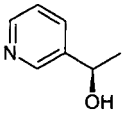
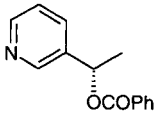
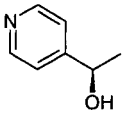
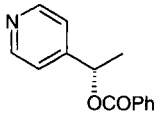
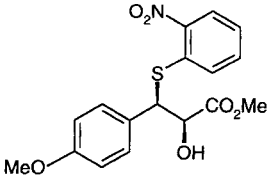
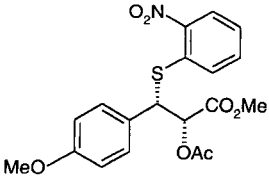
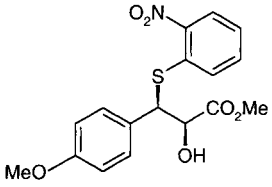
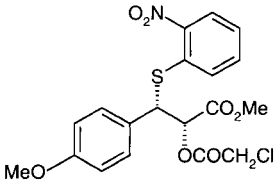
 97 % ee, 42 % yield, PCL	**163a** [68]	 86 % ee, 51 % yield, PCL	**163b** [68]
 95 % ee, 42 % yield, CAL-B	**164a** [69]	 –, –	**164b** [69]
 91 % ee, –, CRL 48 % conversion	**165a** [70]	 97 % ee, –	**165b** [70]
 83 % ee, –, CRL 49 % conversion	**166a** [70]	 80 % ee, –	**166b** [70]
 84 % ee, –, CRL 43 % conversion	**167a** [70]	 65 % ee, –	**167b** [70]
 >99 % ee, 29 %yield, PCL/Celite 98 % ee, 44 % yield, PCL/ENTP-4000 (prepolymer)	**168a** [71]	 48 % ee, 67 % yield 81 % ee, 52 % yield	**168b** [71]

Table 11.1-15. (cont.).



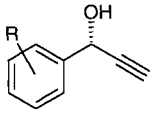
168a [71]

95 % ee, 49 % yield, PCL/ENTP-4000 (prepolymer)



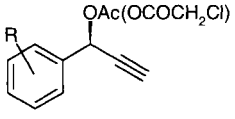
168b [71]

94 % ee, 48 % yield



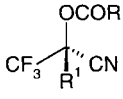
169a [72]

R = H, 2-Me, 3-Me, 4-Me, 3-F, 4-F, 4-CN
80–97 % ee, 30–50 % yield, PSL



169b [72]

80–99 % ee, 30–50 % yield



	R	R^1	
95 % ee, 23 % yield, CRL	Me	Me	**170a** [73]
99 % ee, 37 % yield, CRL	*n*-Bu	Me	**170b** [73]
97 % ee, 36 % yield, CRL	*n*-Pent	Me	**170c** [73]
99 % ee, 30 % yield, CRL	*n*-Hept	Me	**170d** [73]
99 % ee, 40 % yield, CRL	Me	Et	**170e** [73]

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secondary alcohols of the aryl alkyl or dialkyl type are accessible but also those containing all kinds of functional groups in the various positions. An inspection of Tables 11.1-15 and 11.1-13 reveals that in cases where an alkoxycarbonyl group is present as well as the secondary hydroxyl group, two possibilities for enantiomer-differentiation may exist, hydrolysis of the acylated alcohol or hydrolysis of the carboxylic acid ester. Changing the acyl group from acetate to butyrate, chloroacetate, ethylthioacetate or hexadecanoate may have a beneficial effect on the enantioselectivity of the hydrolysis. The use of chloroacetates in many cases facilitates the separation of the ester and the alcohol formed. A series of cyanohydrin acetates have been prepared. Isolation of the cyanohydrin itself is usually not possible because of the alkaline pH. With *Alcaligenes* sp. lipase, which has its pH optimum between 4 and 5, isolation of the cyanohydrin acetate **33b** as well as the cyanohydrin **33a** becomes possible.

Enantiomer separation of α-benzyloxy ketones can be accomplished via lipase-catalyzed enantiomer-differentiating hydrolysis of the corresponding enol esters with formation of a mixture of the resulting ketone and the unchanged enol ester (**94a,b**).

α-Acetoxysulfides (**102–108**), α-acetoxyethers (**140–146**) and α-acetoxyphosphonates (**152–153**) (Table 11.1-15) are useful substrates for lipases too.

Acylated alcohols and alcohols of Table 11.1-15 which can be obtained with other hydrolases as such or of opposite configuration are contained in Tables 11.1-20 and 11.1-22.

A broad structural range of racemic secondary mono-, bi- and tricyclic acylated alcohols are substrates in lipase-catalyzed enantiomer-differentiating hydrolysis as the examples **1–90** of Table 11.1-16 reveal. A large number of *cis*- and *trans*-cycloalkanols bearing a functional group in 2-position (**1–20**, **25**, **26**, **58–62**) is thereby available in enantiomerically pure form. Enantiomer selectivity in the case of cyclic allylic alcohols where the double bond bears no other substituent in the α-position is frequently low. Through a temporary substrate modification such as mono- or dibromination, enantiomerically pure cyclic allylic alcohols may also be obtained in these cases (**51**, **52**).

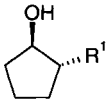
Prochiral diketones or racemic ketones, like enol esters, are also amenable to a hydrolase-catalyzed asymmetric transformation. The enol acetates and ketones **63** and **64**, respectively, may be obtained by *Pseudomonas cepacia* lipase-catalyzed and *Candida cylindracea* lipase-catalyzed hydrolysis of the corresponding racemic enol esters or prochiral bis enol ester, respectively, with high enantioselectivity and yield.

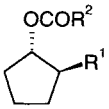
A variety of allylic monocyclic alcohols (**50**, **54**, **56**, **57**, **68–70**, **77–79** and **81**) (Table 11.1-16) have been obtained mainly by *Pseudomonas cepacia* lipase-catalyzed hydrolysis. The planar chiral [2.2]paracyclophane **87** was readily resolved by two different lipases, yielding both enantiomers in almost enantiomerically pure form.

The *Candida cylindracea* lipase-catalyzed, *Candida rugosa* lipase-catalyzed and cholesterol esterase-catalyzed hydrolyses of acetates **88b–102b** are examples of the utilization of a remote phenolic ester group as the site of enzymatic attack. For such cases, cholesterol esterase seems to be particularly well suited.

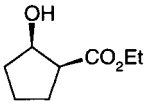
Acylated alcohols and alcohols of Table 11.1-16 which can be obtained with other

Table 11.1-16. Lipase-catalyzed enantiomer-differentiating hydrolysis of esters of racemic cyclic secondary and tertiary alcohols in aqueous solution (PFL *Pseudomonas fluorescens* lipase, PSL *Pseudomonas* sp. lipase, CCL *Candida cylindracea* lipase, ABL *Arthrobacter* sp. lipase, PCL *Pseudomonas cepacia* lipase, CRL *Candida rugosa* lipase, CE cholesterol esterase).



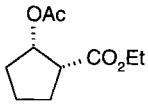


R^1	R^2			lipase		
OAc	Me	≥99 % ee, 30 % yield	**1a**	PFL	30 % ee, 65 % yield	**1b** [1]
CO_2Et	Me	≥99 % ee, 42 % yield	**2a**	PFL	90 % ee, 50 % yield	**2b** [1]
N_3	*n*-Pr	92 % ee, 44 % yield	**3a**	PFL	≥98 % ee, –	**3b** [2]



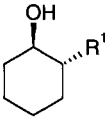
4a [1]

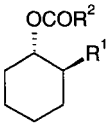
>99 % ee, 43 % yield, PFL



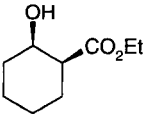
4b [1]

95 % ee, 42 % yield



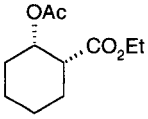


		R^1	R^2	lipase		
5a	≥99 % ee, 33 % yield	OAc	Me	PFL	48% ee, 51 % yield	**5b** [3]
6a	84 % ee, 48 % yield	OAc	Me	PSL	94 % ee, 41 % yield	**6b** [4]
7a	≥99 % ee, 41 % yield	CO_2Et	Me	PFL	55 % ee, 59 % yield	**7b** [3]
8a	96 % ee, 40 % yield	N_3	*n*-Pr	CCL	≥98 % ee, –	**8b** [2]
9a	≥98 % ee, 40 % yield	NO_2	*n*-Pr	CCL	85 % ee, –	**9b** [2]
10a	93 % ee, 40 % yield	CN	*n*-Pr	CCL	93 % ee, –	**10b** [2]
11a	≥98 % ee, 38 % yield	CN	*n*-Pr	PSL	95 % ee, –	**11b** [2]
12a	95 % ee, 44 % yield	Ph	CH_2Cl	PSL	97 % ee, 43 % yield	**12b** [4]
13a	≥95 % ee, 47 % yield	$PhCH_2$	Me	PSL	≥95 % ee, 45 % yield	**13b** [4]
14a	98 % ee, 45 % yield	OMe	Me	PSL	96 % ee, 49 % yield	**14b** [4]
15a	≥99 % ee, 42 % yield	OPh	Me	PSL	96 % ee, 45 % yield	**15b** [4]



16a [3]

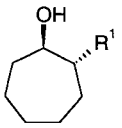
>99 % ee, 32 % yield, PFL

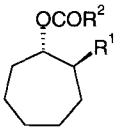


16b [3]

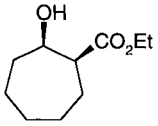
70 % ee, 63 % yield

Table 11.1-16. (cont.).



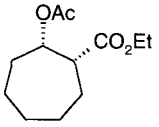


		R^1	R^2	lipase		
17a	≥99 % ee, 45 % yield	OAc	Me	PFL	55 % ee, 55 % yield	**17b** [3]
18a	≥99 % ee, 38 % yield	CO_2Et	Me	PSL	68 % ee, 58 % yield	**18b** [3]
19a	89 % ee, 40 % yield	N_3	*n*-Pr	CCL	91 % ee, –	**19b** [3]



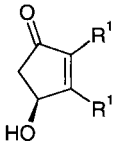
20a [3]

>99 % ee, 36 % yield, PFL



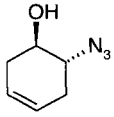
20b [3]

45 % ee, 64 % yield



acetate was not isolated

99 % ee, –, ABL	$R^1 = CH_2C{\equiv}CH$, $R^2 = Me$	**21** [5–7]
98 % ee, –, ABL	$R^1 = CH_2CH{=}CH_2$' $R^2 = Me$	**22** [5–7]
79 % ee, –, ABL	$R^1 = CH_2C{\equiv}CH$, $R^2 = H$	**23** [5–7]
30 % ee, –, ABL	$R^1 = R^2 = H$	**24** [5–7]
	20–50 % conversion	

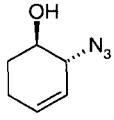


25a [2]

88 % ee, 40 % yield, CCL

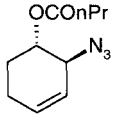
25b [2]

≥98 % ee, –



26a [2]

≥98 % ee, 40 % yield, CCL



26b [2]

94 % ee, –

Table 11.1-16. (cont.).

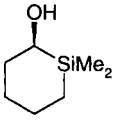
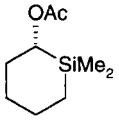
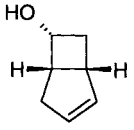
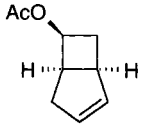
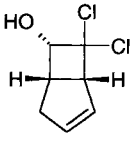
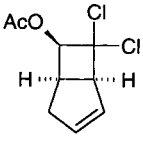
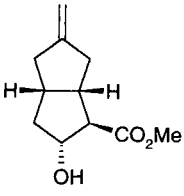
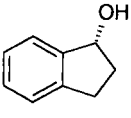
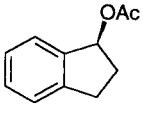
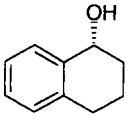
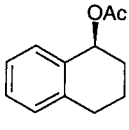
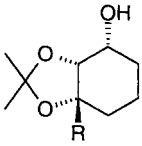
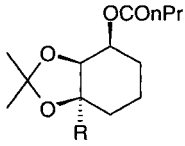
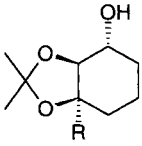
	**27a** [8]		**27b** [8]
95 % ee, 27 % yield, CCL		57 % ee, 50 % yield	
	**28a** [9, 10]		**28b** [9, 10]
≥98 % ee, 46 % yield, PFL		≥98 % ee, – (further hydrolysis of the acetate)	
	**29a** [10]		**29b** [10]
81 % ee, –, PFL		82 % ee, –	
	**30** [3]		
≥99 % ee, 13 % yield, PFL		acetate was not isolated	
	**31a** [11]		**31b** [11]
≥99 % ee, 46 % yield, PSL		≥99 % ee, 47 % yield	
	**32a** [11]		**32b** [11]
≥99 % ee, 47 % yield, PSL		≥99 % ee, 47 % yield	

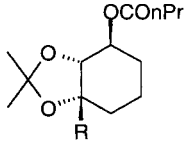
Table 11.1-16. (cont.).



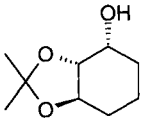


≥95 % ee, 43 % yield, CCL	R = H	**33a** [12]	≥95 % ee, 40 % yield	**33b** [12]
≥95 % ee, 40 % yield, CCL	R = Me	**34a** [12]	44 % ee, 69 % yield	**34b** [12]

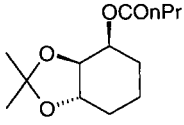




31 % ee, 48 % yield, CCL	R = H	**35a** [12]	36 % ee, 46 % yield	**35b** [12]
84 % ee, 37 % yield, CCL	R = Me	**36a** [12]	77 % ee, 50 % yield	**36b** [12]



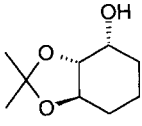
37a [12]



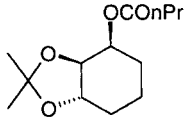
37b [12]

≥95 % ee, 35 % yield, CCL

≥95 % ee, 50 % yield



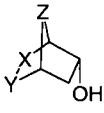
38a [12]

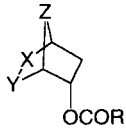


38b [12]

≥95 % ee, 35 % yield, CCL

≥95 % ee, 48 % yield

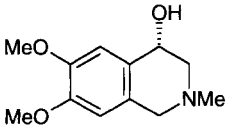




		X-Y	Z	R		
39a	90 % ee, –	CH=CH	CH_2	Me	≥96 % ee, –	**39b** [13]
40a	88 % ee, –	CH=CH	CH_2	*n*-Pr	89 % ee, –, PSL	**40b** [14]
40a	97 % ee, –	CH=CH	CH_2	*n*-Pr		**40b** [14]
41a	93 % ee, –	CH=CH	O	*n*-Pr	≥97 % ee, –	**41b** [15]
42a	75 % ee, –	CH_2-CH_2	CH_2	Me	52 % ee, –	**42b** [11, 14]
43a	94 % ee, –		CH_2	*n*-Pr	≥97 % ee, –	**43b** [11, 14]

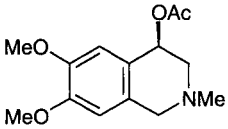
Table 11.1-16. (cont.).

44a	85 % ee, –		CH_2	*n*-Pr	83 % ee, –	**44b** [17]
45a	≥97 % ee, –		O	*n*-Pr	85 % ee, –	**45b** [15]
46a	22 % ee, – all CCL		CH_2	*n*-Pr	14 % ee, –	**46b** [14]



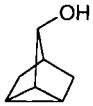
47a [16]

93 % ee, 44 % yield, CCL



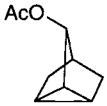
47b [16]

94 % ee, 40 % yield



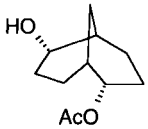
48a [17]

96 % ee, –
(further hydrolysis of reacylated alcohol), CCL



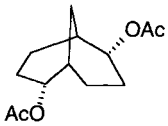
48b [17]

95 % ee, –
(further hydrolysis of acetate)



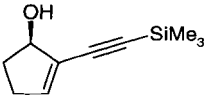
49a [18]

81 % ee, 36 % yield, CCL



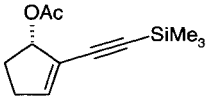
49b [18]

95 % ee, 46 % yield



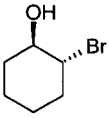
50a [19]

≥99 % ee, 46 % yield, PSL



50b [19]

≥99 % ee, 43 % yield



51a [20]

≥98 % ee, 46 % yield, PCL

51b [20]

≥98 % ee, 46 % yield

Table 11.1-16. (cont.).

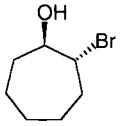
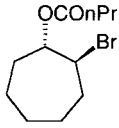
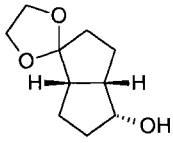
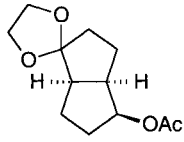
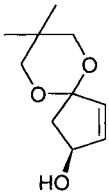
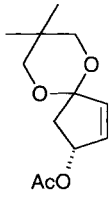
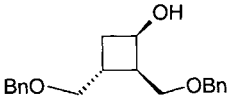
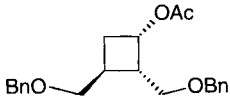
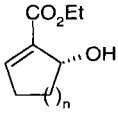
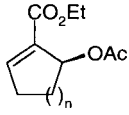
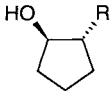
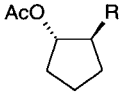
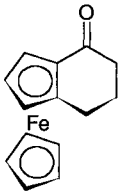
	**52a** [20]		**52b** [20]
≥98 % ee, 41 % yield, PFL		≥98 % ee, 45 % yield	
	**53a** [21]		**53b** [21]
≥95 % ee, 43 % yield, PFL		99 % ee, 49 % yield	
	**54a** [22]		**54b** [22]
≥95 % ee, 34 % yield, PSL		≥95 % ee, 72 % yield	
	**55a** [23]		**55b** [23]
≥99 % ee, 48 % yield, PCL		≥99 % ee, 50 % yield	
			
99 % ee, 44 % yield, PCL	n = 1 **56a** [24]	100 % ee, 45 % yield	**56b** [24]
100 % ee, 43 % yield, PCL	n = 2 **57a** [24]	91 % ee, 52 % yield	**57b** [24]
			
	R		
93 % ee, 45 % yield, PSL	Ph **58a** [25]	≥98 % ee, 42 % yield	**58b** [25]
90 % ee, 43 % yield, PSL	$PhCH_2$ **59a** [25]	93 % ee, 45 % yield	**59b** [25]

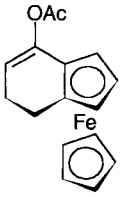
Table 11.1-16. (cont.).

84 % ee, 45 % yield, PSL	PhO	**60a** [25]	≥98 % ee, 45 % yield	**60b** [25]
80 % ee, 43 % yield, PSL	$PhCH_2O$	**61a** [25]	≥98 % ee, 40 % yield	**61b** [25]
51 % ee, 38 % yield, PSL	OAc	**62a** [25]	46 % ee, 45 % yield	**62b** [25]



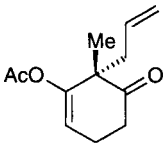
63a [26]

24 % ee, 71 % yield, PCL



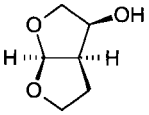
63b [26]

≥99 % ee, 20 % yield



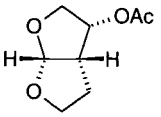
64 [27]

≥98 % ee, 80 % yield CCL



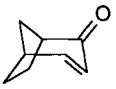
65a [28]

90 % ee, 34 % yield, PCL



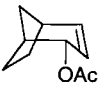
65b [28]

94 % ee, 40 % yield



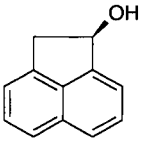
66a [29]

>99 % ee, 37 % yield, CAL-B (in the presence of $PdCl_2(MeCN)_2$ and air)



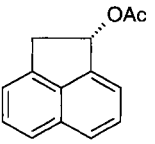
66b [29]

>99 % ee, 42 % yield



67a [30]

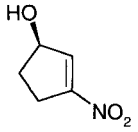
99 % ee, 35 % yield, PFL



67b [30]

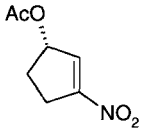
99 % ee, 31 % yield

Table 11.1-16. (cont.).



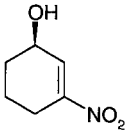
68a [31]

42 % ee, 34 % yield, PCL



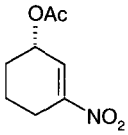
68b [31]

88 % ee, 45 % yield



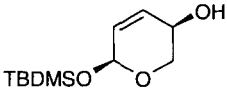
69a [31]

>99 % ee, 32 % yield, PCL



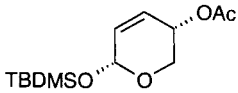
69b [31]

>99 % ee, 43 % yield



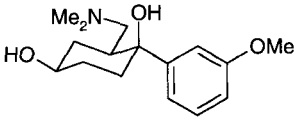
70a [32]

92 % ee, 50 % yield, PSL
87 % ee, 40 % yield, PCL



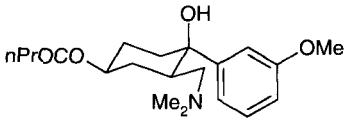
70b [32]

97 % ee, 44 % yield
99 % ee, 39 % yield



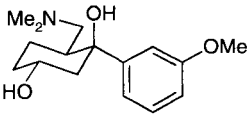
71a [33]

89 % ee, –, CRL, 28 % conversion



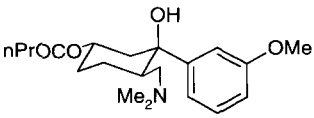
71b [33]

37 % ee, –



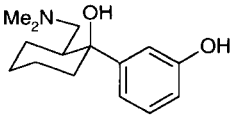
72a [33]

>98 % ee, 40 % yield, CRL



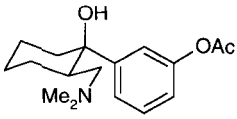
72b [33]

>98 % ee, 40 % yield



73a [33]

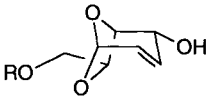
90 % ee, 45 % yield, CRL

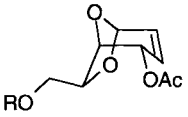


73b [33]

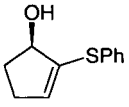
58 % ee, 46 % yield
33 % conversion
>99 % ee, 35 % yield
60 % conversion

Table 11.1-16. (cont.).



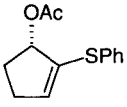


R				
2-Me-naphthyl	>99 % ee, 46 % yield, PCL	**74a** [34]	>99 % ee, 46 % yield	**74b** [34]
CH_2Ph	>99 % ee, 48 % yield, PCL	**75a** [34]	>99 % ee, 49 % yield	**75b** [34]
TBDMS	>99 % ee, 48 % yield, PCL	**76a** [34[	>99 % ee, 48 % yield	**76b** [34]



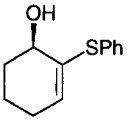
77a [35]

100 % ee, 48 % yield, PCL



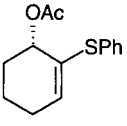
77b [35]

100 % ee, 46 % yield



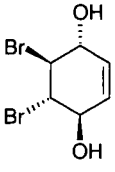
78a [35]

100 % ee, 45 % yield, PCL

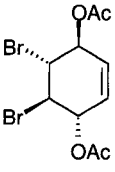


78b [35]

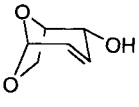
100 % ee, 48 % yield



90 % ee, 47 % yield, PCL **79a** [36a]
>99 % ee, 38 % yield, PPL, after recrystallization **79a** [36b]

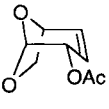


>98 % ee, 26 % yield **79b** [36a]
>99 % ee, 38 % yield, after recrystallization **79b** [36b]



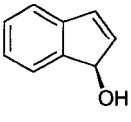
80a [37]

97 % ee, 51 % yield, PSL



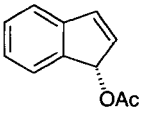
80b [37]

98 % ee, 48 % yield



81a [38]

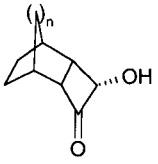
94 % ee, 46 % yield, PCL

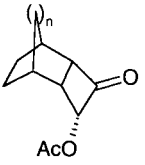


81b [38]

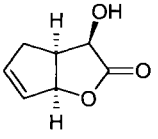
>99 % ee, 45 % yield

Table 11.1-16. (cont.).



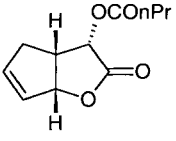


n				
1	>99 % ee, 42 % yield, PCL	**82a** [39]	>99 % ee, 40 % yield	**82b** [39]
2	>99 % ee, 44 % yield, PCL	**83a** [39]	>99 % ee, 48 % yield	**83b** [39]



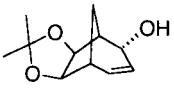
84a [40]

>99 % ee, 30 % yield, PFL



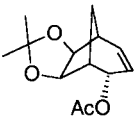
84b [40]

–, –



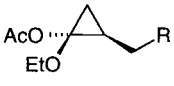
85a [41]

>99 % ee, 49 % yield, PCL



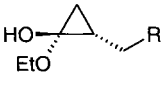
85b [41]

>99 % ee, 51 % yield



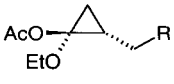
86a [42]

R = *n*-Bu, *n*-C_5H_{11}, *n*-C_6H_{13}, Ph
95–98 % ee, 17–35 % yield, PCL



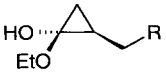
86b [42]

unstable



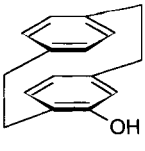
***ent*-86a** [42]

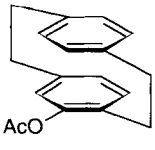
R = *n*-Bu, *n*-C_5H_{11}, *n*-C_6H_{13}, Ph
>99 % ee, 32–49 % yield, CAL-B



***ent*-86b** [42]

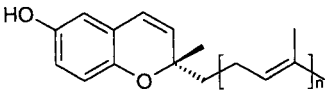
unstable

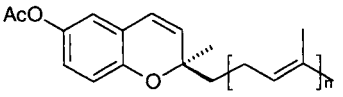




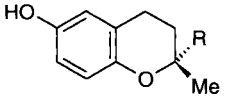
>98 % ee, 46 % yield, CCL	**87a** [43]	>98 % ee, 43 % yield	**87b** [43]
90 % ee, 51 % yield, CRL	**87a** [43]	>99 % ee, 44 % yield	**87b** [44]

Table 11.1-16. (cont.).



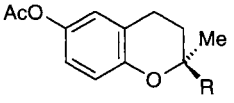


n			
1	13 % ee, 55 % yield, CCL	**88a** [45]	95 % ee, 13 % yield **88b** [45]
2	20 % ee, 65 % yield, CCL	**89a** [45]	98 % ee, 20 % yield **89b** [45]



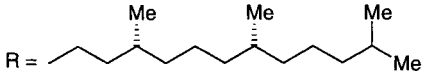
90a [46]

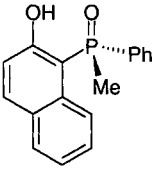
55 % ee, 50 % yield, CE



90b [46]

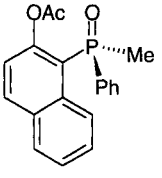
76 % ee, 42 % yield, CE





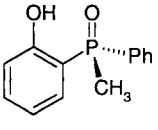
91a [47]

90 % ee, 48 % yield, CRL
99 % ee, 42 % conversion
CE (porcine pancreas),
sodium taurocholate



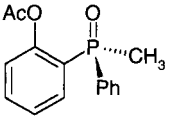
91b [47]

88 % ee, 45 % yield
61 % ee, 42 % conversion
CE (porcine pancreas),
sodium taurocholate



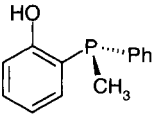
92a [47]

53 % ee, 52 % conversion
CE (porcine pancreas), sodium
taurocholate



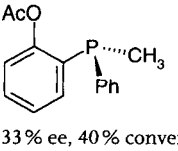
92a [47]

49 % ee, 52 % conversion
CE (porcine pancreas), sodium
taurocholate



93a [47]

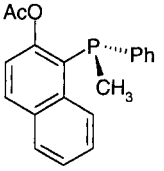
49 % ee, 40 % conversion
CE (porcine pancreas), sodium
taurocholate



93b [47]

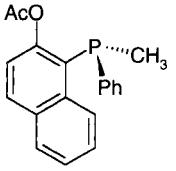
33 % ee, 40 % conversion
CE (porcine pancreas), sodium
taurocholate

Table 11.1-16. (cont.).



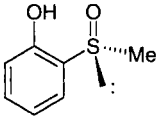
94a [47]

43 % ee, 51 % conversion
CE (porcine pancreas), sodium taurocholate



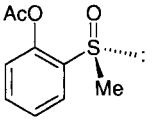
94b [47]

44 % ee, 51 % conversion
CE (porcine pancreas), sodium taurocholate



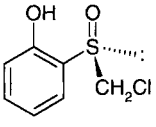
95a [48]

E = 10–15, CE (porcine pancreas),
sodium taurocholate



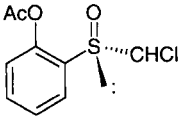
95b [48]

E = 10–15, CE (porcine pancreas),
sodium taurocholate



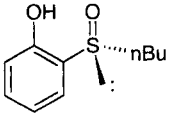
96a [48]

E = 4.6, CE (porcine pancreas),
sodium taurocholate



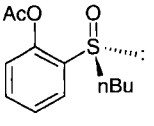
96b [48]

E = 4.6, CE (porcine pancreas),
sodium taurocholate



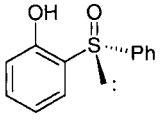
97a [48]

E = 14, CE (porcine pancreas),
sodium taurocholate



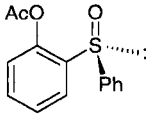
97b [48]

E = 14, CE (porcine pancreas),
sodium taurocholate



98a [48]

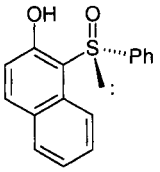
E = 10, CE (porcine pancreas),
sodium taurocholate



98b [48]

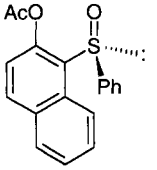
E = 10, CE (porcine pancreas),
sodium taurocholate

Table 11.1-16. (cont.).



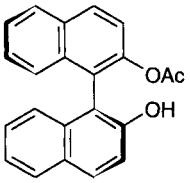
99a [48]

E = 19, CE (porcine pancreas), sodium taurocholate



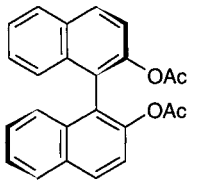
99b [48]

E = 19, CE (porcine pancreas), sodium taurocholate



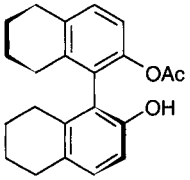
100a [48]

E > 400, CE (porcine pancreas), sodium taurocholate



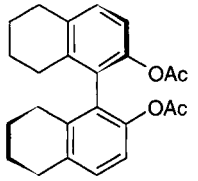
100b [48]

E > 400, CE (porcine pancreas), sodium taurocholate



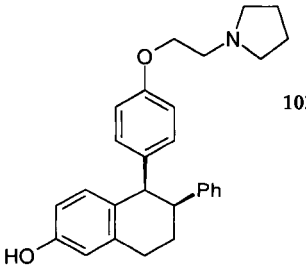
101a [48]

E > 10, CE (porcine pancreas), sodium taurocholate



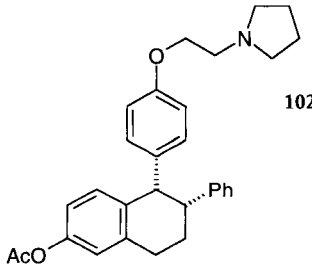
101b [48]

E > 10, CE (porcine pancreas), sodium taurocholate



102a [49]

83% ee, 51% conversion
CE (*Pseudomonas flurorescens*)
51% ee, 35% conversion
CE (porcine pancreas)



102b [49]

80% ee, 51% conversion
CE (*Pseudomonas flurorescens*)
96% ee, 35% conversion
CE (porcine pancreas)

1 Z.-F. Xie, H. Suemune, K. Sakai, *J. Chem. Soc., Chem. Commun.* **1987**, 838.
2 H. Hönig, P. Seufer-Wasserthal, F. Fülöp, *J. Chem. Soc., Perkin Trans.* 1 **1989**, 2341.
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47 A. N. Serreqi, R. J. Kazlauskas, *J. Org. Chem.* **1994**, *59*, 7609.
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hydrolases as such or of opposite configuration are contained in Tables 11.1-6 and 11.1-16.

11.1.1.2
Formation of Carboxylic Esters

11.1.1.2.1 Lipases

Of the many hydrolases known, only the lipases, subtilisin and to some extent α-chymotrypsin, pig liver esterase, and thermolysin[64a] show a sufficiently high

catalytic activity in organic solvents of low water content to be of practical value for asymmetric synthesis through acylation of prochiral or racemic alcohols, alcoholysis of prochiral or racemic acylated alcohols and prochiral anhydrides, and cyclization of racemic hydroxy carboxylic acids. Lipases, as stated previously, are unique for organic synthesis, since they exhibit not only a high catalytic activity in water or in two-phase systems composed of water and a water-immiscible organic solvent or the liquid substrate, but most importantly also in water-miscible or immiscible organic solvents of low water content. This allows for the attainment of favorable equilibria not only in asymmetric hydrolysis but also in esterification reactions.

In the formation of carboxylic esters in an anhydrous organic solvent, its hydrophobicity and the water activity have a major influence on the reaction[30, 36, 134]. Hence, the organic solvent used can significantly influence the selectivity of a lipase-catalyzed enantiotopos- or enantiomer-differentiating reaction. Furthermore, the acyl donor may influence reactivity and selectivity.

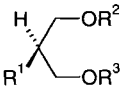
Lipases are most advantageously used for the acylation of prochiral diols or racemic alcohols and for the alcoholysis of racemic acylated alcohols. Generally, through acylation of a prochiral diol or racemic alcohol in an organic solvent such as diethyl ether, diisopropyl ether, *tert*-butyl methyl ether, tetrahydrofuran, dichloromethane, pentane, hexane, toluene or *tert*-pentyl alcohol with acylating reagents such as vinyl acetate, vinyl butyrate, vinyl propionate, vinyl laurate, vinyl palmitate, vinyl chloroacetate, isopropenyl acetate, oxime esters, ethyl acetate, ethyl propionate, trifluoroethyl butyrate, trichloroethyl butyrate, trifluoroethyl acetate, ethyl octanoate, ethyl methoxy acetate, ethyl thiooctanoate, acetic anhydride, succinic anhydride or 2-phenyloxazolin-5-one and hydrolysis of the corresponding prochiral diacetate (dipropionate, dichloroacetate) or racemic acetate (chloroacetate) in water or in water and a water-immiscible organic solvent, access to both enantiomers of the corresponding monoacetate and alcohol, respectively, is provided with one enzyme (Tables 11.1-10 to 11.1-12 and 11.1-18). This is because of the same enantiotopic group and enantiomer recognition shown in general by the enzyme in both reactions (Scheme 11.1-12), and favorable opposite equilibria.

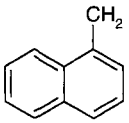
In many cases vinyl acetate, isopropenyl acetate, ethyl acetate and propionyl acetate not only serve as acylating reagents but also as solvents.

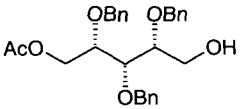
For the acylation of prochiral diols, *ee* values of monoacetates (about 90%) can be raised considerably in most cases by a higher degree of conversion at the expense of a lower chemical yield to the point where an enantiomer-differentiating formation of the diacetate can take place (Scheme 11.1-11, Figure 11.1-1), because in most cases the enzyme preferentially catalyzes the acylation of the minor enantiomer. The enantioselectivity and thermostability of lipases is frequently enhanced in organic solvents of low water content. A minimum amount of water is required for the catalytic activity of the lipase. In most cases lipase preparations with a residual water content of approximately 1% in anhydrous organic solvents are employed. Frequently in organic solvents of low water content the thermostability of lipases is much higher than that in aqueous solution[36].

The use of lipase in other forms than lyophilized powders, as for example on different kinds of solid supports, entrapped in sol-gel materials or as CLECs, has the

Table 11.1-17. Lipase-catalyzed enantiotopos-differentiating acylation of prochiral acyclic diols in organic solvents (CCL *Candida cylindracea* lipase, PFL *Pseudomonas fluorescens* lipase, PPL pig pancreas lipase, CVL *Chromobacterium viscosum* lipase, PSL *Pseudomonas* sp. lipase, RJL *Rhizomucor javanicus* lipase, ANL *Aspergillus niger* lipase, CAL *Candida antarctica* lipase, not specified, PCL *Pseudomonas cepacia* lipase, CRL *Candida rugosa* lipase).

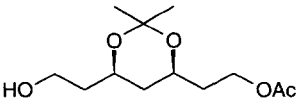


	R^1	R^2	R^3	Lipase	Acyl donor	ee (%)	yield (%)	Ref.
1	Me	H	Ac	PFL	vinyl acetate	60	70	[1]
2	CH_2=CH-CH_2	Ac	H	PFL	vinyl acetate	81	89	[1]
3	CH_2=CH-$(CH_2)_2$	Ac	H	PPL[a]	ethyl acetate	90	70	[2]
4	Ph	Ac	H	PPL[a]	ethyl acetate	92	98	[2]
5	CH_2Ph	Ac	H	PPL[a]	ethyl acetate	13	90	[2]
5	CH_2Ph	Ac	H	PFL	vinyl acetate	≥94	100	[3]
				PFL	vinyl acetate	97	96	[1]
6		Ac	H	PFL	vinyl acetate	86	93	[3]
				PFL	vinyl acetate	90	95	[1]
7	*i*-Pr	Ac	H	PFL	vinyl acetate	61	85	[1]
8	*c*-C_6H_{11}	Ac	H	PPL[a]	ethyl acetate	58	90	[2]
9	*c*-$C_6H_{11}CH_2$	Ac	H	PPL[a]	ethyl acetate	10	90	[2]
10	Cbz	Ac	H	PPL	vinyl acetate	97	77	[4]
11	OCH_2Ph	H	Ac	PFL	isopropenyl acetate	96	53	[4]
11	OCH_2Ph	H	Ac	PFL	vinyl acetate	92	92	[5]
11	OCH_2Ph	H	Ac	PFL	phenyl acetate	90	88	[5]
12	OEt	H	Ac	PFL	phenyl acetate	90	90	[5, 6]



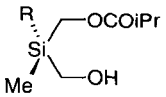
13 [7]

≥95 % ee, 70 % yield, CCL vinyl acetate



14 [8]

≥98 % ee, 51 % yield, PFL vinyl acetate



R = Ph	70 % ee, 80 % yield, CCL methyl isobutyrate	**15** [9]	70 % ee, 50 % yield, CVL methyl isobutyrate	***ent*-15** [9]
R = *n*-octyl	75 % ee, 63 % yield, CCL methyl isobutyrate	**16** [9]	76 % ee, 70 % yield, CVL methyl isobutyrate	***ent*-16** [9]

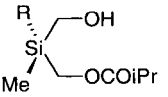
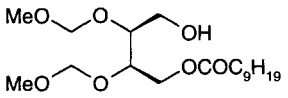
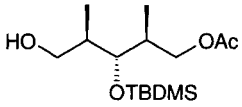


Table 11.1-17. (cont.).



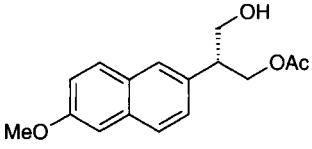
17 [10]

95 % ee, 90 % yield, PPL
$C_9H_{19}COCH_2CCl_3$



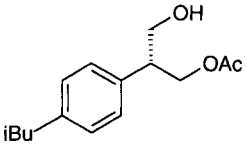
18 [11]

97 % ee, 94 % yield, CRL,
vinyl acetate



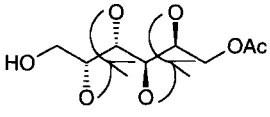
19 [12]

89 % ee, 80 % yield, PPL,
vinyl acetate



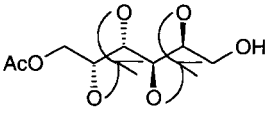
20 [12]

>99 % ee, 76 % yield, PPL,
vinyl acetate



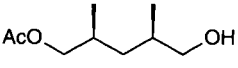
21 [13]

98 % ee, 86 % yield, PFL,
vinyl acetate



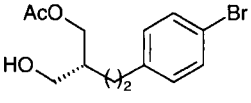
***ent*-21** [13]

98 % ee, 75 % yield, PCL,
vinyl acetate



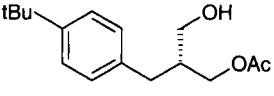
22 [14]

92 % ee, 55 % yield, PPL,
methyl acetate



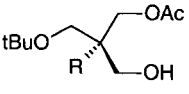
23 [15]

94 % ee, 86 % yield, PPL,
vinyl acetate

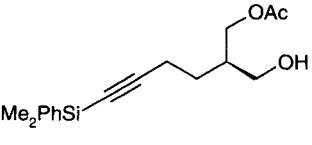


25 [16]

99 % ee, 86 % yield, PSL,
vinyl acetate

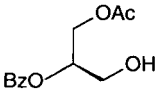


R = H, 91 % ee, 97 % yield, PFL, vinyl acetate **26** [17]
R = Me, 88 % ee, 80 % yield, PFL, vinyl acetate **27** [17]



28 [18]

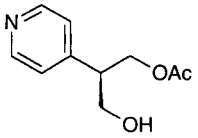
>99 % ee, 98 % yield, PFL, vinyl acetate
95 % ee, 98 % yield, PCL, vinyl acetate



29 [19]

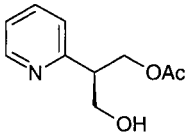
96 % ee, 63 % yield, PPL,
vinyl acetate

Table 11.1-17. (cont.).



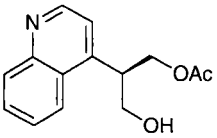
30 [20]

96 % ee, 84 % yield, PPL, vinyl acetate

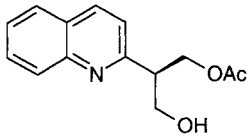


31 [20]

98 % ee, 81 % yield, PPL, vinyl acetate

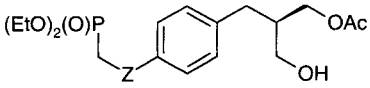


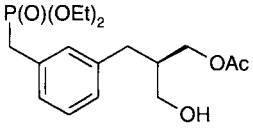
84 % ee, 21 % yield, ANL, vinyl acetate **32** [21]
98 % ee, 42 % yield, CAL, vinyl acetate ***ent*-32** [21]



33 [21]

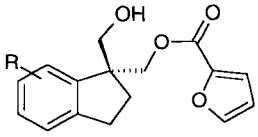
97 % ee, 46 % yield, PPL, vinyl acetate





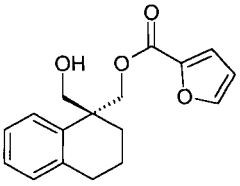
Z		
CH_2	99 % ee, 97 % yield, PCL, vinyl acetate	**34** [22]
CF_2	99 % ee, 82 % yield, PCL, vinyl acetate	**35** [22]

87 % ee, 95 % yield, PCL, vinyl acetate **36** [22]



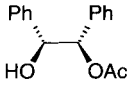
37 [23]

85–92 % ee, 35–78 % yield, CRL, 1-ethoxyvinyl 2-furoate abs. config. not determined



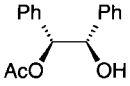
38 [23]

61 % ee, 84 % yield, CRL, 1-ethoxyvinyl 2-furoate abs. config. not determined



39 [24]

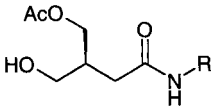
90 % ee, 20 % yield, RJL, vinyl acetate



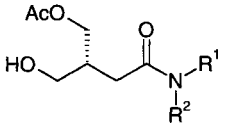
***ent*-39** [24]

90 % ee, 28 % yield, CCL, vinyl acetate

Table 11.1-17. (cont.).



R		
Me	>99 % ee, 26 % yield, PCL	**40** [25]
Et	phenyl acetate 94 % ee, 43 % yield, PCL phenyl acetate	**40** [25]



R^1	R^2		
Me	Me	97 % ee, 42 % yield, PCL vinyl acetate	**41** [25]
Me	Me	96 % ee, 77 % yield, PCL phenyl acetate	**41** [25]
Et	Et	96 % ee, 52 % yield, PCL vinyl acetate	**42** [25]
Et	Et	84 % ee, 43 % yield, PCL phenyl acetate	**42** [25]

a purified PPL

1 K. Tsuji, Y. Terao, K. Achiwa, *Tetrahedron Lett.* **1989**, *30*, 6189.
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advantage of easy recovery by filtration and reuse. Furthermore, these lipases have higher stability, and, most importantly, their activity and selectivity are often much higher than with the lyophilized powders.

One should bear in mind, however, that in nearly all cases lipase preparations are used, which contain, as well as a large amount of mostly unspecified material such as proteins, carbohydrates and solid support materials, only a minor amount of the lipase and in several case even additional mostly unidentified hydrolases. The solid material contained in the crude lipase preparation may have an important stabilizing function in organic solvents, in which the lipase preparation is insoluble. Crude lipase preparations supplied commercially contain up to 7% of water. Drying the solid material in vacuum may reduce the water content. Acylating reagents such as vinyl acetate and isopropenyl acetate are very useful since they allow for an extreme equilibrium position in acylation because of the tautomerization of the vinyl and isopropenyl alcohol formed to acetaldehyde and acetone, respectively. The possible harmful effect of acetaldehyde on the enzyme with the crude lipase preparation used poses practically no problem in most cases because the low price of the enzyme enables relatively large amounts of it to be used. Synthetically, lipase-catalyzed acylations are convenient to carry out and, in contrast to the corresponding hydrolyses, catalysts are easy to recover and can be reused.

A series of alkyl, alkoxy or acylamino 1,3-proanediol derivatives substituted in 2-position have been subjected to lipase-catalyzed acylation, and the monoacetates (**1–12**, **19**, **20**, **23–38**, **40–42**) were obtained with moderate to high enantiomeric excess (Table 11.1-17). For the monoacetates **1–12**, reactions with and in ethyl acetate are usually slower than those with and in vinyl acetate. As in the hydrolysis of the corresponding diacetates, much higher selectivities were recorded with the yet unidentified carboxyl esterase from crude pig pancreas lipase. An excellent lipase for the enantioselective acylation of 3-benzyloxy-1,3-propane diol is *Pseudomonas fluorescens* lipase, which gives high selectivity with vinyl acetate, isopropenyl acetate and ethyl acetate. By carrying the acylation further, to a certain extent to the diacetate, the enantiomerically pure monoacetate should be obtainable.

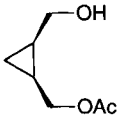
Sterically demanding substituents in 2-position such as in **20**, **25**, **26**, **28**, **29** and **34–36** guarantee high enantioselectivity and yield for the monoacetates.

Sila propanediol derivatives (**15**, **16**), and butanediol, pentanediol, hexanediol and heptanediol derivatives (**17**, **18**, **21**, **22**) (Table 11.1-17) have also been prepared.

Monoacetates of Table 11.1-17 which can be obtained with other hydrolases as such or of opposite configuration are contained in Tables 11.1-4 and 11.1-10.

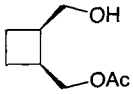
Cyclic dimethanol derivatives have been extensively studied not only in lipase-catalyzed hydrolysis (Table 11.1-11) but also in lipase-catalyzed enantioselective acylation for synthetic and mechanistic reasons (**1–16**, **20**, **30**, **32**, **33**, **37**, **40**, **45**, **47–53**, **57–62**, **66**, **72**) (Table 11.1-18). Generally, enantioselectivities in acylation of the diol and hydrolysis of the corresponding diacetate yielding enantiomeric com-

Table 11.1-18. Lipase-catalyzed enantiotopos-differentiating acylation of prochiral cyclic diols in organic solvents (PPL pig pancreas lipase, PFL *Pseudomonas fluorescens* lipase, PCL *Pseudomonas cepacia* lipase, CCL *Candida cylindracea* lipase, MSL *Mucor* sp. lipase, CVL *Chromobacterium viscosum* lipase, GCL *Geotrichum candidum* lipase, CRL *Candida rugosa* lipase, MML *Mucor miehei* lipase, CAL-B *Candida antarctica* B lipase, LIP *Pseudomonas* sp. lipase-Toyobo).



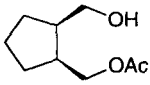
1 [1]

≥95 % ee, 82 % yield, PFL, vinyl acetate



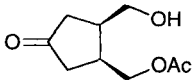
2 [1]

88 % ee, 87 % yield, PFL, vinyl acetate
≥95 % ee, 82 % yield, PFL, ethyl acetate



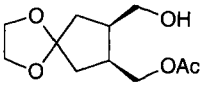
3 [1]

≥95 % ee, 85 % yield, PFL vinyl acetate



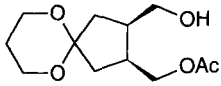
4 [2, 3]

94 % ee, 64 % yield, PPL, vinyl acetate



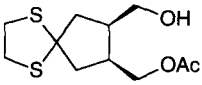
5 [2, 3]

25 % ee, 52 % yield, PPL, vinyl acetate



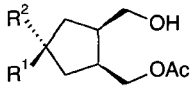
6 [2, 3]

≥98 % ee, 87 % yield, PPL, vinyl acetate



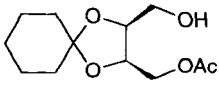
7 [2, 3]

≥95 % ee, 87 % yield, PPL
vinyl acetate



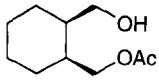
8 [2, 3]

R^1	R^2	PPL, vinyl acetate
OH	H	98 % ee, 92 % yield
Ph_3CO	H	45 % ee, 72 % yield
H	Cl	≥96 % ee, 84 % yield
H	SPh	≥96 % ee, 85 % yield
H	SO_2Ph	68 % ee, 69 % yield
H	N_3	≥95 % ee, 86 % yield



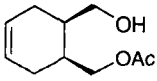
9 [3]

77 % ee, 91 % yield, PPL, vinyl acetate
≥98 % ee, 78 % yield, PFL, vinyl acetate
9 % ee, 71 % yield, PFL, acetic anhydride



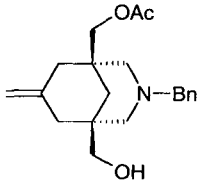
10 [1]

7 % ee, 44 % yield, PFL, vinyl acetate



11 [1]

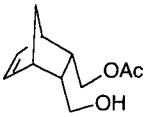
80 % ee, 60 % yield, PFL, vinyl acetate



12 [4]

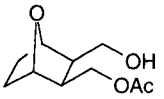
100 % ee, 32 % yield, CCL, vinyl acetate

Table 11.1-18. (cont.).



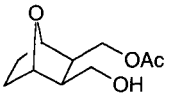
13 [5]

80 % ee, 80 % yield, GCL, vinyl acetate, CH_2Cl_2
95 % ee, 72 % yield, GCL, vinyl acetate, Et_2O



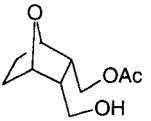
14 [6]

99 % ee, 68 % yield, PPL, ethyl acetate
99 % ee, 92 % yield, PPL, vinyl acetate



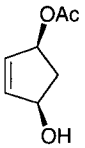
15 [6]

96 % ee, 71 % yield, CCL, isopropenyl acetate
76 % ee, 70 % yield, CCL, vinyl acetate



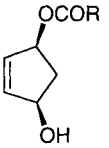
16 [6]

8 % ee, 38 % yield, PPL, vinyl acetate
87 % ee, 72 % yield, CCL, vinyl acetate



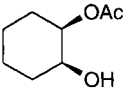
17 [7–11]

98 % ee, –, PFL, vinyl acetate
98 % ee, 52 % yield, PPL, vinyl acetate
>95 % ee, 48 % yield, pancreatin, trichloroethyl acetate
>99 % ee, 50 % yield, PPL, trichloroethyl acetate
>99 % ee, 65 % yield, pancreatin, vinyl acetate
94 % ee, 85 % yield, MSL, vinyl acetate



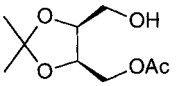
18 [11a]

R = *n*-Pr, *n*-C_7H_{15}' CH_2Cl
>99–80 % ee, 58–39 % yield, pancreatin, trichloroethyl alkanoate



19 [2, 3]

84 % ee, –, PPL, vinyl acetate

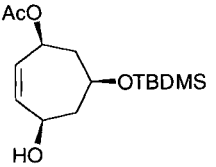


20 [12]

95 % ee, 80 % yield, PFL, vinyl acetate

21 [13]

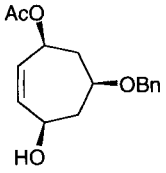
59 % ee, 60 % yield, PSL, vinyl acetate



22 [14, 15]

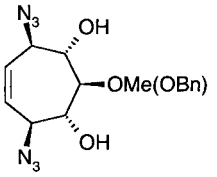
≥95 % ee, 95 % yield, PCL, isopropenyl acetate

Table 11.1-18. (cont.).



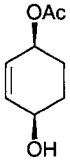
23 [16]

≥97 % ee, 89 % yield, PCL, vinyl acetate



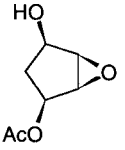
24 [16]

no acylation with vinyl acetate and several lipases



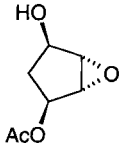
25 [17]

95 % ee, 51 % yield, PCL, vinyl acetate



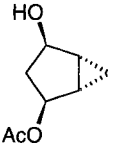
26 [18]

≥99 % ee, 38 % yield, PPL
94 % ee, 75 % yield, PCL, vinyl acetate



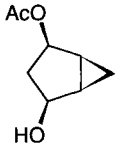
27 [18]

2 % ee, 60 % yield, MSL, vinyl acetate



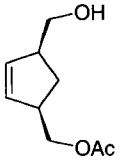
28 [18]

≥99 % ee, 94 % yield, PPL
≥99 % ee, 87 % yield, PCL, vinyl acetate



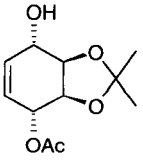
29 [18]

86 % ee, 75 % yield, PPL, vinyl acetate



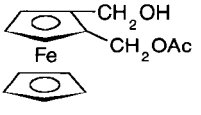
30 [19]

97 % ee, 68 % yield, CCL
18 % ee, 57 % yield, PPL, vinyl acetate



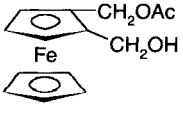
31 [20]

≥95 % ee, 90 % yield, PCL, isopropenyl acetate



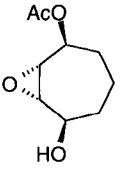
32 [21]

100 % ee, 80 % yield, PCL, vinyl acetate



33 [21]

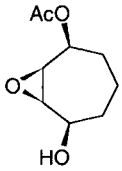
100 % ee, 80 % yield, CVL, vinyl acetate



34 [22]

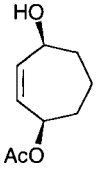
≥98 % ee, 46 % yield, PCL, isopropenyl acetate

Table 11.1-18. (cont.).



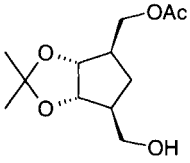
35 [22]

84 % ee, 92 % yield, PCL, vinyl acetate



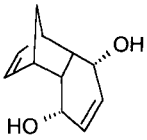
36 [22]

17 % ee, –, PCL, isopropenyl acetate



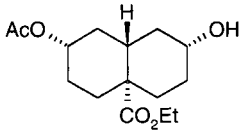
37 [23]

≥99 % ee, 81 % yield, PFL, vinyl acetate



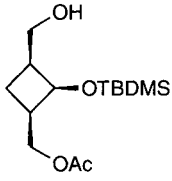
38 [24]

≥99 % ee, 87 % yield, PCL, vinyl acetate



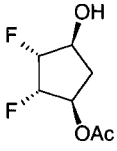
39 [25]

≥99 % ee, 96 % yield, PSL, vinyl acetate



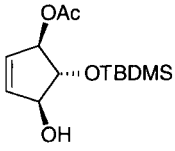
40 [26]

95 % ee, 98 % yield, CCL, vinyl acetate



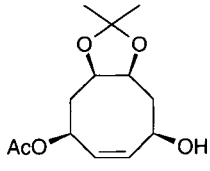
41 [27]

98 % ee, 70 % yield, CRL, vinyl acetate
91 % ee, 64 % yield, PCL, vinyl acetate



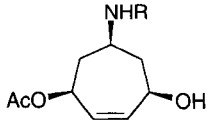
42 [28]

>99 % ee, 99 % yield, PSL, vinyl acetate



43 [29]

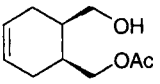
>98 % ee, 90 % yield, PCL, isopropenyl acetate



44 [30]

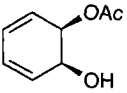
R = Cbz: >98 % ee, 91 % yield, PCL, isopropenyl acetate
R = Boc: >98 % ee, 92 % yield, PCL, isopropenyl acetate

Table 11.1-18. (cont.).



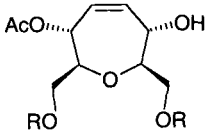
45 [31]

>99 % ee, 88 % yield, PPL, ethyl acetate

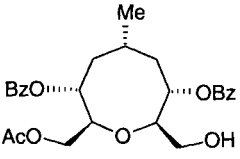


46 [32]

>98 % ee, 89 % yield, PCL, vinyl acetate
95 % ee, 93 % yield, MML, vinyl acetate
70 % ee, 89 % yield, CAL-B, vinyl acetate

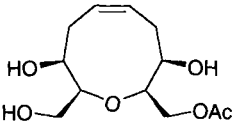


R		
TBDMS	>99 % ee, 58 % yield, PCL, vinyl acetate	47 [33]
	>99 % ee, 65 % yield, PSL, vinyl acetate	
TIPS	>99 % ee, 77 % yield, PSL, vinyl acetate	48 [33]
CH_2Ph	>99 % ee, 81 % yield, PSL, vinyl acetate	49 [33]



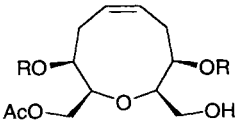
50 [33]

92 % ee, 92 % yield, PSL, vinyl acetate



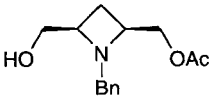
51 [33]

91 % ee, 82 % yield, PSL, vinyl acetate



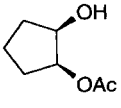
52 [33]

R = 4-$MeOC_6H_4CH_2$
94 % ee, 76 % yield, PSL, vinyl acetate



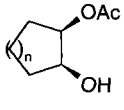
53 [34]

>99 % ee, 65 % yield, PPL, vinyl acetate



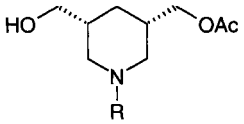
54 [35]

88 % ee, 73 % yield, PSL, vinyl acetate

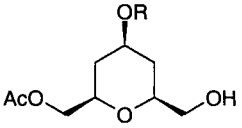


n = 2: >98 % ee, 94 % yield, MML, vinyl acetate — 55 [35]
n = 3 : 80 % ee, 85 % yield, PSL, vinyl acetate — 56 [35]

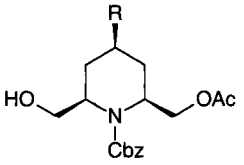
Table 11.1-18. (cont.).



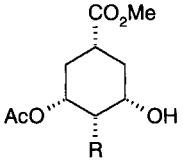
R = Boc: >98 % ee, 74 % yield, PFL, vinyl acetate **57** [36]
R = Cbz: >98 % ee, 78 % yield, PFL, vinyl acetate **58** [36]



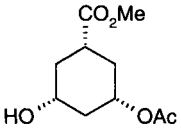
R = TBDMS: >98 % ee, 70 % yield, CAL-B, isopropenyl acetate **59** [37]
R = MOM: >95 % ee, 68 % yield, CAL-B, isopropenyl acetate **60** [37]



R = H: 95 % ee, 80 % yield, CAL-B, vinyl acetate **61** [38]
R = OMOM: 96 % ee, 83 % yield, CAL-B, vinyl acetate **62** [38]

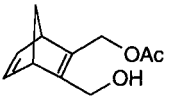


R = Me: >99 % ee, 42 % yield, PSL, vinyl acetate **63** [39]
R = Me: 93 % ee, 50 % yield, PSL, vinyl acetate **64** [39]
R = Et: >99 % ee, 53 % yield, PSL, vinyl acetate



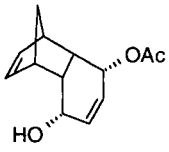
65 [39]

>99 % ee, 97 % yield, PPL, vinyl acetate



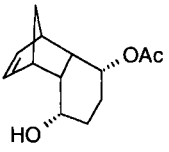
66 [40]

>95 % ee, 89 % yield, PSL, vinyl acetate



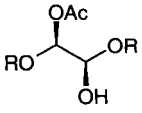
67 [41]

>99 % ee, 88 % yield, LIP, vinyl acetate



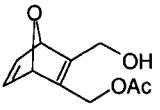
68 [41]

>99 % ee, 82 % yield, LIP, vinyl acetate



R		
Bn	93 % ee, 93 % yield, LIP, vinyl acetate	**69** [42]
4-$MeOC_6H_4CH_2$	84 % ee, 85 % yield, LIP, vinyl acetate	**70** [42]
2-NaphthylCH_2	93 % ee, 93 % yield, LIP, vinyl acetate	**71** [42]

Table 11.1-18. (cont.).

	72 [43]
92 % ee, 47 % yield, PPL, vinyl acetate >98 % ee, 75 % yield, PCL 90 % ee, 73 % yield, PSL	

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pounds differ but not to a large extent (Tables 11.1-11 and 11.1-18). In many cases the enantioselectivity of acylation is higher than that of the hydrolysis. Acylation of the three-, four- and five-membered dimethanol derivatives proceeds uniformly with the same enantiotopic group recognition to the monoacetates **1–8** with good to high enantioselectivity and yield. Acylation of the cyclohexanoid dimethanol system is erratic, giving **10** with low enantioselectivity and low yield. The cyclohexenoid system **11** however is obtained with the same lipase with good enantioselectivity. Acylation of cyclopentanoid dimethanol derivatives with a functional group in 4-position by pig pancreas lipase has been intensively investigated (**4–8**). The enantioselectivity can be influenced (**5** and **6**) by the choice of the appropriate protecting group. The heterocyclic dimethanol monoacetate **9**, which is a derivative of the parent compound *meso*-butane tetrol, is obtained with high enantioselectivity by *Pseudomonas fluorescens* lipase instead of pig pancreas lipase. Acylation of *meso-exo*-oxa-norbornane dimethanol with pig pancreas lipase and with *Candida cylindracea* lipase provides access to both enantiomeric monoacetates **14** and **15**. A further example of the attainment of both enantiomers by changing the lipase is provided by the acylation of 1,2-bis(hydroxymethyl)ferrocene with vinyl acetate catalyzed either by *Pseudomonas cepacia* lipase which gives the (*S*)-enantiomer **32** or by *Chromobacterium viscosum* lipase which gives the (*R*)-enantiomer **33**. Acylation catalyzed by lipases is, as in the case of the hydrolysis of the corresponding acetates, not restricted to substrates containing primary hydroxyl groups, as demonstrated by the successful synthesis of the monoacetates **17–19**, **22–29**, **31**, **34–36**, **38**, **39**, **41–44**, **46**, **63–65**. These examples give a good illustration of the scope of lipases as catalysts. Comparison of the bicyclo[3.1.0]cyclohexane derivatives **28** and **29** shows that changing the configuration of the cyclopropane ring is accompanied by a switch of enantiotopos-selectivity under identical reaction conditions.

Lipases are the hydrolases of choice for the kinetic enantiomer separation of racemic primary, secondary and tertiary alcohols through acylation. Acylation of the racemic alcohols is complementary to the hydrolysis or alcoholysis of the corresponding esters.

Monoacetates of Table 11.1-18 which can be obtained with other hydrolases as such or of opposite configuration are contained in Tables 11.1-3, 11.1-7, 11.1-9 and 11.1-11.

A large number of enantiomerically pure primary alcohols carrying additional nitrogen, oxygen and sulfur functionalities can be prepared by lipase-catalyzed enantiomer-differentiating acylation with the usual acylating reagents (**1–130**) (Table 11.1-19). Most remarkably, a series of primary alcohols whose chiral center bears only alkyl or alkenyl groups (**23–30**) has been obtained with high enantioselectivity through *Pseudomonas fluorescens* lipase-catalyzed acylation with vinyl acetate in dichloromethane. For the attainment of chiral primary alcohols, lipase-catalyzed acylation seems to be more efficient in terms of selectivity and yield than lipase-catalyzed hydrolysis of the corresponding esters. A comparison of Tables 11.1-19 and 11.1-14 shows that enantiomer-differentiating hydrolysis of acetates and enantiomer-differentiating acylation of the corresponding alcohols catalyzed by one and the same lipase are complementary. Enantiomer-differentiating acylation with succinic

Table 11.1-19. Lipase-catalyzed enantiomer-differentiating acylation of racemic acyclic primary alcohols in organic solvents (PPL pig pancreas lipase, PFL *Pseudomonas fluorescens* lipase, PCL *Pseudomonas cepacia* lipase, CCL *Candida cylindracea* lipase, MML *Mucor miehei* lipase, PSL *Pseudomona* sp. lipase, CAL-B *Candida antarctica* B lipase, CAL *Candida antarctica* lipase, not specified, CLL *Candida lipolytica* lipase, SML *Serratia marcensens* lipase, HLL *Humicola lanuginosa* lipase).

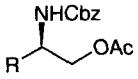
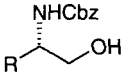
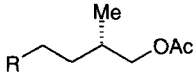
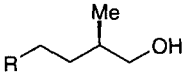
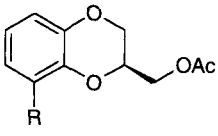
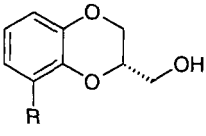
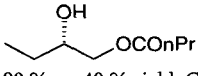
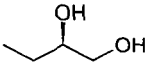
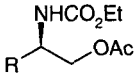
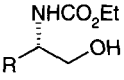
				
R				
Me	73 % ee, –	**1a**	85 % ee, –	**1b** [1]
Et	78 % ee, –	**2a**	83 % ee, –	**2b** [1]
n-Pr	99 % ee, –	**3a**	99 % ee, –	**3b** [1]
n-Bu	95 % ee, –	**4a**	95 % ee, –	**4b** [1]
	all PPL, ethyl acetate			
				
R = PhS	98 % ee, –	**5a**	98 % ee, –	**5a** [2]
R = $PhSO_2$	98 % ee, –	**6a**	98 % ee, –	**6a** [2]
	all PCL, vinyl acetate		60 % conversion	
	40 % conversion			
				
R = H	26 % ee, –, PCL, vinyl acetate	**7a**	≥99 % ee, 37 % yield	**7b** [3]
R = OMe	81 % ee, 43 % yield, PCL, acetic anhydride	**8a**	83 % ee, 44 % yield	**8b** [4]
		**9a** [5]		**9b** [5]
90 % ee, 40 % yield, CCL tributyrin			89 % ee, 36 % yield	
				
R = Me	90 % ee, 31 % yield	**10a**	≥95 % ee, 30 % yield	**10b** [6]
R = Et	≥95 % ee, 31 % yield	**11a**	92 % ee, 32 % yield	**11b** [6]
	all PPL, ethyl acetate			

Table 11.1-19. (cont.).

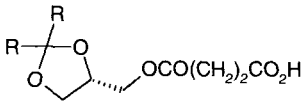
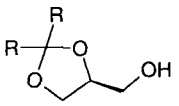
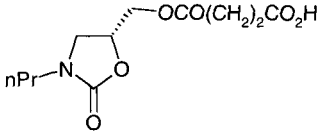
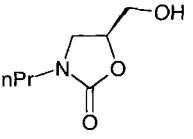
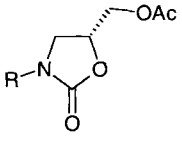
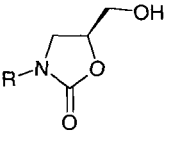
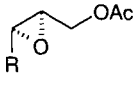
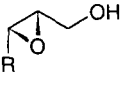
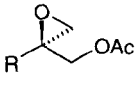
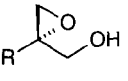
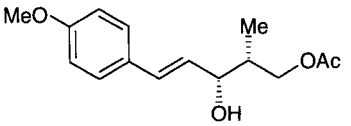
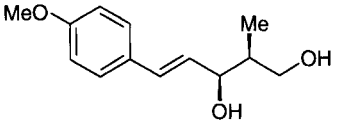
				
R = Me	60 % ee, 40 % yield	**12a**	61 % ee, 40 % yield	**12b** [7]
R = Ph	92 % ee, 41 % yield all PFL, succinic anhydride	**13a**	70 % ee, 32 % yield	**13b** [7]
		**14a** [7]		**14b** [7]
75 % ee, 46 % yield, PFL succinic anhydride			98 % ee, 38 % yield	
		**15a** [8]		**15b** [8]
R = *t*-Bu, *i*-Pr ≥95 % ee, 40–43 % yield, PFL acetic, propionic or butyric anhydride			≥95 % ee, 42–45 % yield	
				
R = $C_{10}H_{21}$	PPL	**16a**	≥95 % ee, 31 % yield	**16b** [9, 10]
R = $(CH_2)_4CHMe_2$	ethyl acetate	**17a**	≥95 % ee, 36 % yield	**17b** [9, 10]
				
R = $PhCH_2$	≥98 % ee, 32 % yield	**18a**	≥98 % ee, 34 % yield	**18b** [11a]
R = C_9H_{19}	96 % ee, 38 % yield	**19a**	96 % ee, 36 % yield	**19b** [11a]
R = Vinyl-$(CH_2)_3$	99 % ee, 38 % yield all PFL, vinyl acetate 40 % conversion	**20a**	98 % ee, 38 % yield 60 % conversion	**20b** [11b]

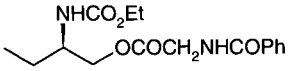
Table 11.1-19. (cont.).



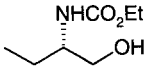
94 % ee, 23 % yield, PSL isopropenyl acetate — **21a** [12]



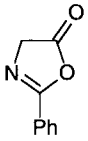
97 % ee, 27 % yield — **21b** [12]

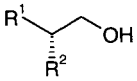


93 % ee, –, MML — **22a** [13]

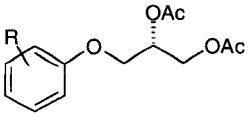


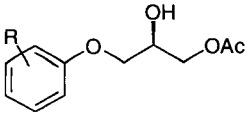
63 % ee, –, — **22b** [13]





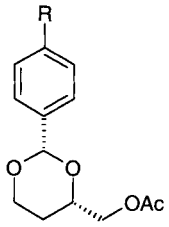
R^1	R^2			
n-Pr	Me	98 % ee, 17 % yield	all PCL	**23** [14]
n-Bu	Me	99 % ee, 22 % yield	vinyl acetate.	**24** [14]
n-Bu	Et	97 % ee, 23 % yield	The	**25** [14]
n-Hex	Me	96 % ee, 20 % yield	corresponding	**26** [14]
$(CH_3)_2CHCH_2$	Me	98 % ee, 26 % yield	acetates were of	**27** [14]
n-Oct	Me	98 % ee, 26 % yield	low ee.	**28** [14]
$CH_3CH{=}CHCH_2$	Me	96 % ee, 33 % yield		**29** [14]
Allyl	Me	97 % ee, 25 % yield		**30** [14]

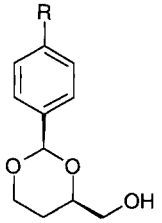




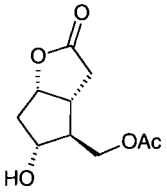
R				
H	79 % ee, 46 % yield	**31a**	85 % ee, 48 % yield	**31b** [15]
2-Me	80 % ee, 48 % yield	**32a**	93 % ee, 45 % yield	**32b** [16]
3-OMe	95 % ee, 47 % yield	**33a**	91 % ee, 49 % yield	**33b** [15]
4-OMe	94 % ee, 52 % yield	**34a**	96 % ee, 48 % yield	**34b** [15]
4-Cl	92 % ee, 49 % yield	**35a**	94 % ee, 48 % yield	**35b** [15]
4-*t*-Bu	93 % ee, 50 % yield	**36a**	99 % ee, 50 % yield	**36b** [15]
	all PCL, vinyl acetate			

Table 11.1-19. (cont.).

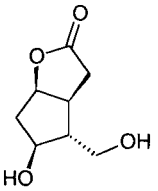




R				
H	82 % ee, 48 % yield	**37a**	≥97 % ee, 39 % yield	**37b** [17]
Me	90 % ee, 46 % yield	**38a**	90 % ee, 48 % yield	**38b** [17]
i-Pr	84 % ee, 47 % yield	**39a**	≥94 % ee, 38 % yield	**39b** [17]
F	79 % ee, 40 % yield	**40a**	90 % ee, 33 % yield	**40b** [17]
OMe	90 % ee, 48 % yield	**41a**	≥98 % ee, 42 % yield	**41b** [17]
	all PSL, vinyl acetate			



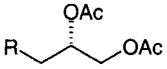
42a [18]

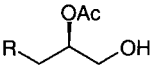


42b [18]

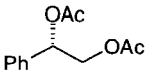
86 % ee, 52 % yield, PCL vinyl acetate

99 % ee, 44 % yield

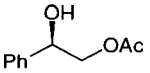




R				
i-Pr	>98 % ee, 37 % yield	**43a**	57 % ee, 53 % yield	**43b** [19]
t-Bu	>98 % ee, 39 % yield	**44a**	81 % ee, 52 % yield	**44b** [19]
Ph	>98 % ee, 34 % yield	**45a**	67 % ee, 57 % yield	**45b** [19]
	all PFL, vinyl acetate			



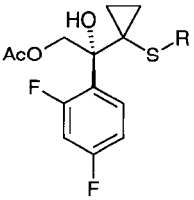
46a [20]



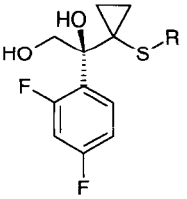
46b [20]

96 % ee, 48 % yield, PCL, vinyl acetate

87 ee, 52 % yield



47a [21]



47b [21]

R = 4-OMe$C_6H_4CH_2$
>99 % ee, 33 % yield, lipase OF (Meito Sangyo), vinyl acetate

R = 4-OMe$C_6H_4CH_2$
–, –

Table 11.1-19. (cont.).

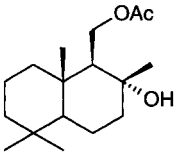
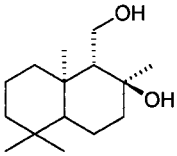
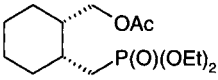
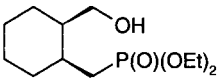
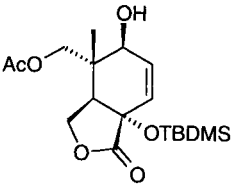
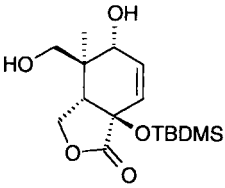
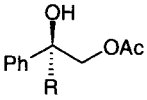
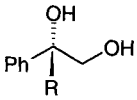
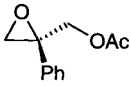
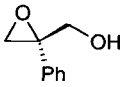
 97 % ee, 20 % yield, PCL vinyl acetate	**48a** [22]	 27 % ee, 76 %yield	**48b** [22]
 97 % ee, 35 % yield, PCL vinyl acetate 93 % ee, 35 % yield, PSL vinyl acetate	**49a** [23]	 62 % ee, 60 % yield >99 % ee, 41 % yield	**49b** [23]
 90 % ee, 51 % yield, CAL-B, isopropenyl acetate	**50a** [24]	 –, 49 % yield.	**50b** [24]
 R = CH_2Ph: >97 % ee, –, PSL vinyl acetate, 50 % conversion	**51a**	 >97 % ee, –	**51b** [25]
R = 4-$MeC_6H_4CH_2$: 94 % ee, –, PSL, vinyl acetate, 49 % conversion	**52a**	90 % ee, –	**52b** [25b]
R = 4-$MeC_6H_4CH_2$: 73 % ee, –, PSL, vinyl acetate, 58 % conversion	**53a**	>99 % ee, –	**53b** [25b]
R = CH_2I: 94 % ee, –, PSL vinyl acetate, 43 % conversion	**54a**	70 % ee, –	**54b** [26]
 94 % ee, –, PSL, vinyl acetate 51 % conversion	**55a** [26]	 >97 % ee, –	**55b** [26]

Table 11.1-19. (cont.).

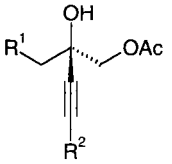
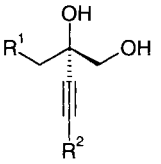
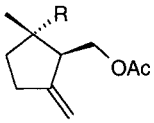
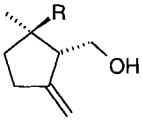
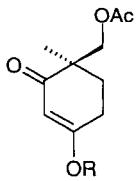
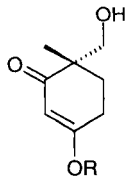
			
R^1 = Cl, R^2= Ph: 95 % ee, –, PPL, vinyl acetate, 41 % conversion	**56a**	67 % ee, –	**56b** [26]
R^1 = I, R^2= Ph: 96 % ee, –, PPL, vinyl acetate, 51 % conversion	**57a**	>97 % ee, –	**57b** [26]
R^1 = I, R^2= CH_2-CH_2Ph: 55 % ee, –, PPL, vinyl acetate, 64 % conversion	**58a**	>97 % ee, –	**58b** [26]
R^1 = I, R^2= $SiMe_3$: 94 % ee, –, PPL, vinyl acetate, 51 % conversion	**59a**	>97 % ee, –	**59b** [26]
R^1 = I, R^2= *n*-Bu: 89 % ee, –, PPL, vinyl acetate, 49 % conversion	**60a**	88 % ee, –	**60b** [26]
R^1 = I, R^2= *n*-Hex: 51 % ee, –, PPL, vinyl acetate, 64 % conversion	**61a**	93 % ee, –	**61b** [26]
R^1 = I, R^2= *t*-Bu: >97 % ee, –, PPL, vinyl acetate, 49 % conversion	**62a**	94 % ee, –	**62b** [26]
			
R = 4-MePh: 72 % ee, 55 % yield, CRL, vinyl acetate	**63a**	>99 % ee, 42 % yield	**63b** [27]
R = Me: 62 % ee, –, PSL, vinyl acetate, 62 % conversion	**64a**	96 % ee, –	**64b** [28]
			
R = Me: 90 % ee, 36 % yield, PCL, vinyl acetate	**65a**	69 % ee, 54 % yield	**65b** [29]
R = Me: 90 % ee, 38 % yield, PSL, vinyl acetate	**66a**	96 % ee, 45 % yield	**66b** [29]
R = MOM: 94 % ee, 40 % yield, PCL, vinyl acetate		75 % ee, 51 % yield	
R = MOM: 97 % ee, 46 % yield, PSL, vinyl acetate		99 % ee, 48 % yield	

Table 11.1-19. (cont.).

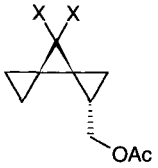
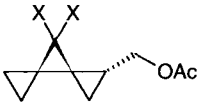
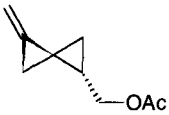
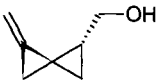
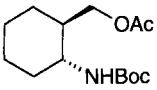
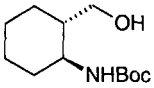
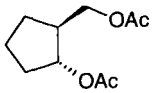
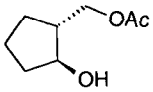
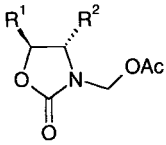
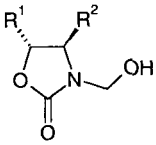
				
X = Cl: >95 % ee, 43 % yield, PCL, vinyl acetate	**67a**		>95 % ee, 37 % yield	**67b** [30]
X = F: >95 % ee, 44 % yield, PCL, vinyl acetate	**68a**		>95 % ee, 33 % yield	**68b** [30]
X = H: >95 % ee, 39 % yield, PCL, vinyl acetate	**69a**		>95 % ee, 33 % yield	**69b** [30]
	**70a** [30]			**70b** [30]
–, 50 % yield, PCL, vinyl acetate			>95 % ee, 43 % yield	
	**71a** [31]			**71b** [31]
91 % ee, 45 % yield, PCL, vinyl butyrate			925 % ee, 38 % yield	
	**72a** [32]			**72b** [32]
90 % ee, 51 % yield, PCL, vinyl acetate			>99 % ee, 48 % yield	
				
R^1	R^2			
Ph	H	73 % ee, 47 % yield, PCL, vinyl propionate	78 % ee, 42 % yield	**73b** [33]
H	Ph	92 % ee, 43 % yield, PCL, vinyl propionate	81 % ee, 48 % yield	**74b** [33]
H	Ph	99 % ee, 44 % yield, PCL, vinyl propionate	74 % ee, 51 % yield	**75b** [33]
H	CH_2Ph	71 % ee, 52 % yield, PCL, vinyl propionate	87 % ee, 43 % yield	**76b** [33]

Table 11.1-19. (cont.).

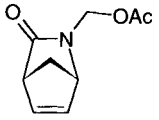
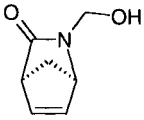
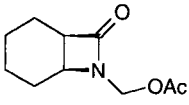
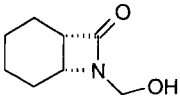
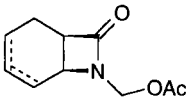
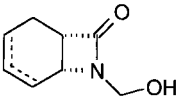
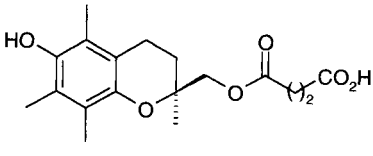
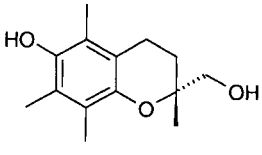
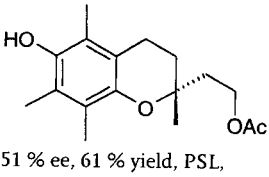
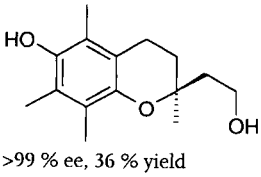
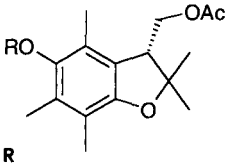
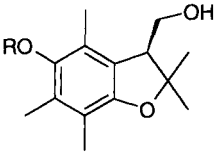
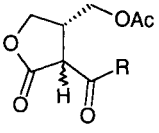
	**77a** [34]		**77b** [34]
94 % ee, 38 % yield, PCL, vinyl acetate 89 % ee, 31 % yield, PSL, vinyl acetate		89 % ee, 40 % yield 91 % ee, 40 % yield	
	**78a** [35]		**78b** [35]
99 % ee, 40 % yield, PCL, vinyl butyrate		97 % ee, 36 % yield	
			
Δ3(4): 99 % ee, 34 % yield, PCL, vinyl butyrate	**79a**	94 % ee, 32 % yield	**79b** [35]
Δ4(5): 99 % ee, 34 % yield, PCL, vinyl butyrate	**80a**	97 % ee, 25 % yield	**80b** [35]
			
96 % ee, 47 % yield, PCL, succinic anhydride	**81a** [36]	–, 42 % yield	**81b** [36]
	**82a** [37]		**81b** [37]
51 % ee, 61 % yield, PSL, vinyl acetate		>99 % ee, 36 % yield	

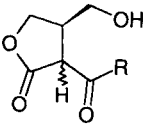
Table 11.1-19. (cont.).



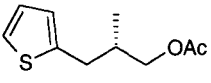


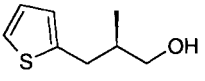
R				
Me	92 % ee, –, CRL, vinyl acetate, 59 % conversion	**83a**	–, –	**83b** [38] **84b** [38]
CH_2Ph	96 % ee, –, CRL, vinyl acetate, 50 % conversion	**84a**	–, –	



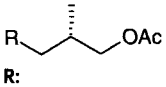


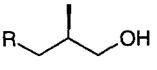
R				
$(CH_2)_3CH(CH_3)_2$	>97 % ee, 52 % yield, CLL, Ac_2O	**85a**	–, 22 % yield	**85b** [39]
n-Pentyl	88 % ee, 49 % yield, CLL, Ac_2O	**86a**	–, 25 % yield	**86b** [39]
n-Bu	>95 % ee, 46 % yield, olipase 4SD (Amano), Ac_2O	**87a**	–, 34 % yield	**87b** [39]
n-Pr	73 % ee, 50 % yield, CLL, Ac_2O	**88a**	–, 38 % yield	**88b** [39]



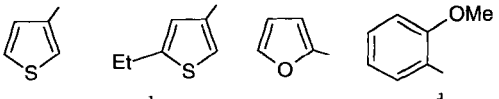


75 % ee, 55 % yield, PFL, vinyl acetate	**89a**	96 % ee, 42 % yield	**89b** [40]
98 % ee, –, PFL, vinyl acetate, 39 % conversion	**89a**	>99 % ee, –, 57 % conversion	**89b** [41]





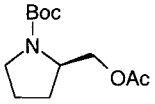
R:



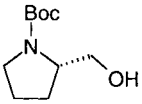
a b c d

R = a: 97 % ee, –, PCL, vinyl acetate, 39 % conversion	**90a**	–, –	**90b** [42]
R = b: 95 % ee, –, PCL, vinyl acetate, 38 % conversion	**91a**	–, –	**91b** [42]
R = c: 97 % ee, –, PCL, vinyl acetate, 31 % conversion	**92a**	–, –	**92b** [42]
R = d: 94 % ee, –, PCL, vinyl acetate, 41 % conversion	**93a**	–, –	**93b** [42]

Table 11.1-19. (cont.).



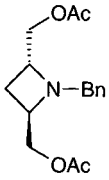
94a [43]



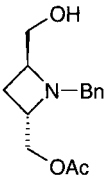
94b [43]

–, –, PCL, vinyl acetate 58 % conversion

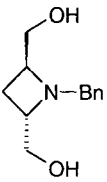
96 % ee, –



95a

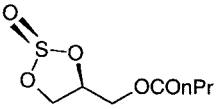


95b

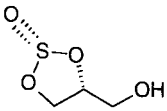


95c

88 % ee, 50 % yield, PCL, vinyl acetate	78 % ee, 21 % yield	>99 % ee, 29 % yield	[44a]
80 % ee, 21 % yield, PPL, vinyl acetate	83 % ee, 26 % yield, for ***ent*-95b**	>99 % ee, 53 % yield	[44b, c]



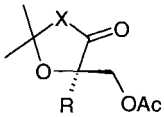
96a [45]

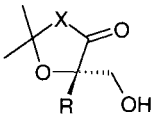


96b [45]

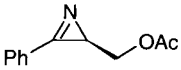
91 % ee, 18 % yield, PCL, vinyl butyrate

24 % ee, 64 % yield

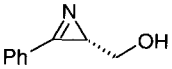




X	R				
O	Ph	>99 % ee, –, CAL, vinyl acetate, 32 % conversion	**97a**	46 % ee, –	**97b** [46]
O	4-Br-Ph	98 % ee, –, CAL, vinyl acetate, 38 % conversion	**98a**	59 % ee, –	**98b** [46]
O	Me	91 % ee, –, PPL, vinyl acetate, 29 % conversion	**99a**	38 % ee, –	**99b** [46]
NMe	Ph	82 % ee, –, CAL, vinyl acetate, 54 % conversion	**100a**	>98 % ee, –	**100b** [46]



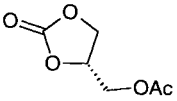
101a [47]



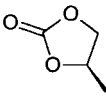
101b [47]

96 % ee, 38 % yield, PCL, vinyl acetate, –50 °C

62 % ee, 60 % yield



102a [48]

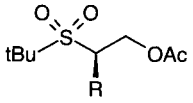


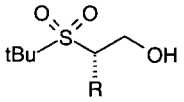
102b [48]

62 % ee, 55 % yield, PFL, vinyl acetate

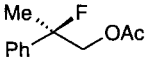
94 % ee, 40 % yield

Table 11.1-19. (cont.).



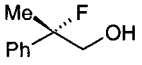


R				
Me	73 % ee, –, CAL-B, vinyl acetate, 57 % conversion	**103a**	92 % ee, –	**103b** [49]
CH_2Ph	81 % ee, –, CAL-B, vinyl acetate, 57 % conversion	**104a**	99 % ee, –	**104b** [49]



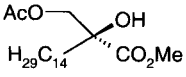
105a [50]

95 % ee, 26 % yield, PCL, vinyl acetate



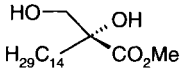
105b [50]

70 % ee, 31 % yield



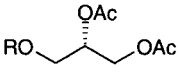
106a [51]

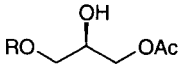
83 % ee, 25 % yield, PFL, vinyl acetate



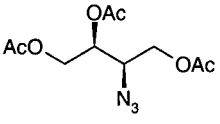
106b [51]

54 % ee, 52 %



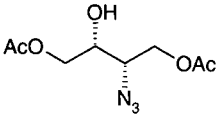


R				
$C_{16}H_{33}$	80 % ee, –, PSL, vinyl acetate, 55 % conversion, 4 °C	**107a**	94 % ee, –	**107b** [52]
$C_{18}H_{37}$	30 % ee, –, PSL, vinyl acetate, 51 % conversion, 4 °C	**108a**	89 % ee, –	**108b** [52]
(9Z)-$C_{18}H_{35}$	24 % ee, –, PSL, vinyl acetate, 61 % conversion, 22 °C	**109a**	93 % ee, –	**109b** [52]



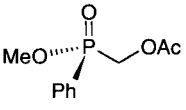
110a [53]

91 % ee, –, PCL, vinyl acetate, 50 % conversion



110b [53]

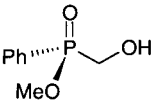
>99 % ee, –



111a [54]

89 % ee, 39 % yield, PFL, vinyl acetate

86 % ee, 44 % yield, PCL, vinyl acetate

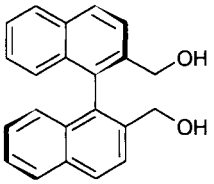


111b [54]

80 % ee, 44 % yield

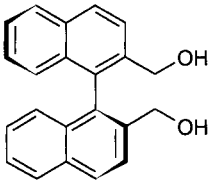
92 % ee, 42 % yield

Table 11.1-19. (cont.).



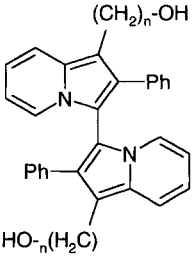
112 [55]

acylated enantiomers
not shown
>99 % ee, 29 % yield, SML,
vinyl hexanoate

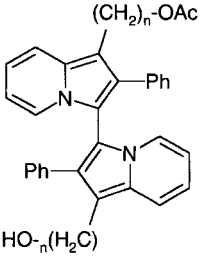


***ent*-112** [55]

acylated enantiomers
not shown
>99 % ee, 45 % yield, HLL,
vinyl hexanoate

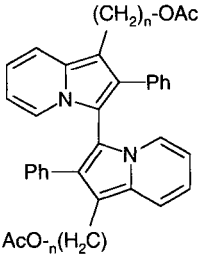


absolute configuration
unknown
n = 2: **113a**
90 % ee, 41 % yield,
CAL-B, vinyl acetate
n = 3: **114a**
>99 % ee, 9 % yield,
PSL, vinyl acetate



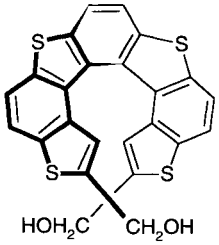
absolute configuration
unknown
113b
35 % ee, 27 % yield

114b
>98 % ee, 18 % yield

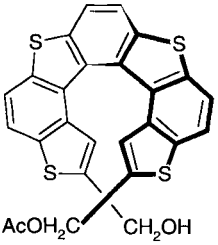


absolute configuration
unknown
113c [56]
99 % ee, 32 % yield

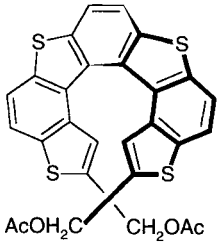
114c [56]
30 % ee, 73 % yield



***(P)*-115a** [57]
98 % ee, 45 % yield,
PCL, vinyl acetate
***(M)*-115a** [57]
92 % ee, 44 % yield,
CAL-B, vinyl acetate

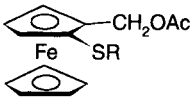


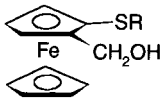
***(M)*-115b** [57]
80 % ee, 38 % yield



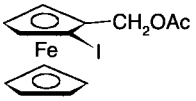
***(M)*-115c** [57]
95 % ee, 13 % yield

Table 11.1-19. (cont.).



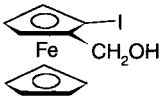


R				
Me	81 % ee, –, MML, vinyl acetate, 46 % conversion 84 % ee, 47, CAL-B, vinyl acetate, 46 % conversion	**116a**	81 % ee, – 83 % ee, 47 % yield	**116b** [58]
Ph	88 % ee, –, CAL-B, vinyl propionate, 45 % conversion	**117a**	72 % ee, –	**117b** [58b]
t-Bu	90 % ee, –, MML, vinyl acetate, 35 % conversion	**118a**	48 % ee, –	**118b** [58b]



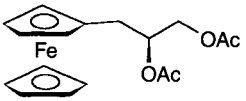
119a [59]

89 % ee, –, CAL-B, vinyl acetate, 52 % conversion



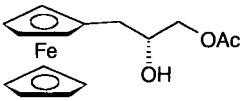
119b [59]

96 % ee, –



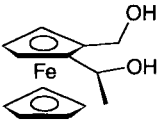
120a [60]

92 % ee, 49 % yield, PCL, vinyl acetate

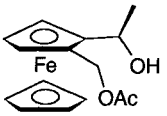


120b [60]

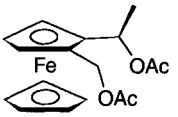
90 % ee, 51 % yield



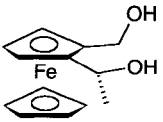
121a [61]
>97 % ee, 22 % yield, PCL, vinyl acetate



121b [61]
10 % ee, 55 % yield

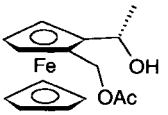


121c [61]
>97 % ee, 18 % yield



122a [61]

>95 % ee, 42 % yield, PCL, vinyl acetate



122b [61]

58 % ee, 59 % yield

Table 11.1-19. (cont.).

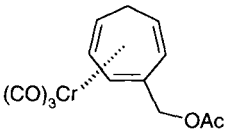
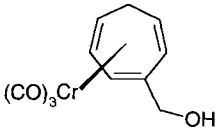
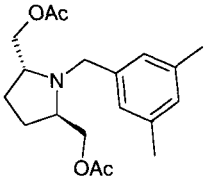
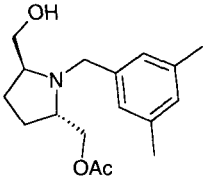
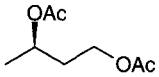
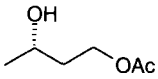
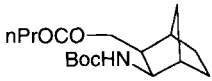
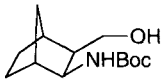
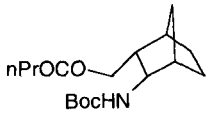
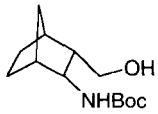
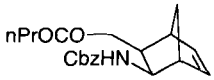
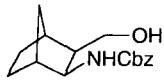
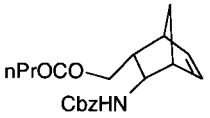
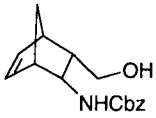
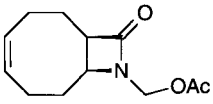
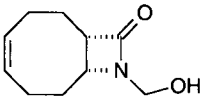
	**123a** [62]		**123b** [62]
75 % ee, 35 % yield, PCL, isopropenyl acetate The two further regioisomers show lower selectivity.		80 % ee, 30 % yield	
	**124a** [63]		**124b** [63]
94 % ee, –, PCL, vinyl acetate, 38 % conversion 97 % ee, –, PFL, vinyl acetate, 38 % conversion		56 % ee, – 60 % ee, –	
	**125a** [64]		**125b** [64]
91 % ee, 44 % yield, CAL-B, vinyl acetate		91 % ee, 44 % yield	
	**126a** [65]		**126b** [65]
89 % ee, 51 % yield, CAL-B, vinyl butyrate		99 % ee, 36 % yield	
	**127a** [65]		**127b** [65]
95 % ee, 18 % yield, PPL, vinyl butyrate, 37 % conversion		96 % ee, 43 % yield, 53 % conversion	
	**128a** [65]		**128b** [65]
95 % ee, 40 % yield, PPL, vinyl butyrate, 44 % conversion		99 % ee, 36 % yield, 56 % conversion	

Table 11.1-19. (cont.).

	**129a** [65]		**129b** [65]
92 % ee, 22 % yield, PPL, vinyl butyrate, 30 % conversion		90 % ee, 32 % yield, 53 % conversion	
	**130a** [66]		**130b** [66]
83 % ee, 44 % yield, PCL, vinyl acetate		95 % ee, 34 % yield	

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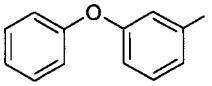
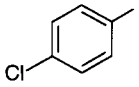
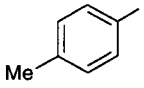
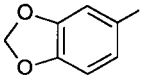
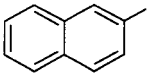
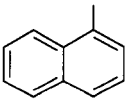
acid anhydride (**12–14**) instead of the usually employed vinyl acetate may facilitate the separation of the remaining substrate and the ester formed because of the carboxyl group in the latter.

Mixed primary secondary diols are very often separated into their enantiomers in a sequential two-step acylation wherein the first step – acylation of the primary hydroxyl group – shows high regio- but very poor enantioselectivity. The useful enantiomer-differentiating step is realized in the second step by acylation of the already monoacylated diol (**31–36, 46, 72, 107–110, 120, 125**). On the other hand, as expected, mixed primary tertiary diols are not acylated at the tertiary hydroxyl group (**47, 48, 51–54, 56–62, 106**).

Regarding the structural diversity of the primary alcohols, which have been successfully resolved, there seems to be almost no limitation, as demonstrated by the axial-chiral diols **112–114**, the ferrocene alcohols **116–122** and the chiral chromium carbonyl complex **123**, and most remarkably the helicenediol **115** (Table 11.1-19).

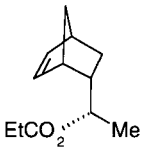
Table 11.1-20. Lipase-catalyzed enantiomer-differentiating acylation of racemic acyclic secondary alcohols in organic solvents (PSL *Pseudomonas* sp. lipase, PPL pig pancreas lipase, PFL *Pseudomonas fluorescens* lipase, PCL *Pseudomonas cepacia* lipase, CCL *Candida cylindracea* lipase, CAL-B *Candida antarctica* B lipase, CAL-A *Candida antarctica* A lipase, LIP *Pseudomonas* sp. lipase-Toyobo, BSL *Burkholderia* sp. lipase, GLL goat liver lipase, RML *Rhizomucor* sp. lipase, CRL *Candida rugosa* lipase).

1 [1, 2]

R	ee (%)	Yield (%)
	89	80
	96	84
	84	83
	91	64
	91	81
	85	88
	70	70

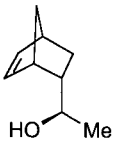
Amberlite IRA-904, aldehyde, acetone cyanohydrin, all PSL, vinyl acetate (dynamic kinetic resolution)

Table 11.1-20. (cont.).



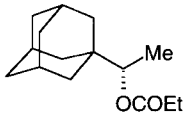
2a [3]

≥95 % ee, –, PPL, $EtCO_2Me$



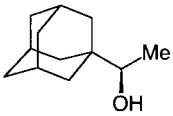
2b [3]

35 % ee, –



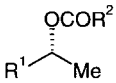
3a [3]

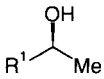
≥98 % ee, –, PPL, $EtCO_2Me$



3b [3]

18 % ee, –





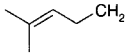
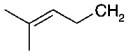
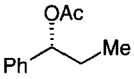
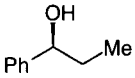
R^1	R^2				
Et	*n*-Pr	93 % ee, 38 % yield, CCL, tributyrin	**4a**	89 % ee, 35 % yield	**4b** [4, 5]
n-C_6H_{13}	*n*-Pr	95 % ee, 35 % yield, PPL, $Cl_3CCH_2OCOnPr$	**5a**	90 % ee, 30 % yield	**5b** [6]
n-C_6H_{13}	*n*-Pr	92 % ee, 41 % yield, CCL, tributyrin	**5a**	95 % ee, 38 % yield	**5b** [7]
n-C_8H_{17}	*n*-Pr	≥99 % ee, 44 % yield, PPL, Cl_3CCH_2OCOPr	**6a**	95 % ee, 44 % yield	**6b** [6]
n-$C_{14}H_{29}$	*n*-Pr	98 % ee, 42 % yield, PPL, Cl_3CCH_2OCOPr	**7a**	≥99 % ee, 43 % yield	**7b** [6]
	Me	–, –, PPL, vinyl acetate	**8a**	≥98 % ee, –	**8b** [7]
	$C_{11}H_{23}$	80 % ee, 38 % yield, PPL, $F_3CCH_2OCOC_{11}H_{23}$	**9a**	≥97 % ee, 43 % yield	**9b** [8]
	n-PR	87 % ee, 31 % yield, PPL, $Cl_3CCH_2OCOnPr$	**10a**	92 % ee, 26 % yield	**10b** [6]
Ph	Me	≥99 % ee, 45 % yield, PSL, vinyl acetate	**11a**	93 % ee, 41 % yield	**11b** [9]
Ph	Me	≥95 % ee, 39 % yield, PCL, acetic anhydride	**11a**	≥95 % ee, 43 % yield	**11b** [10]
$PhCH_2$	Me	≥99 % ee, 30 % yield, PSL, vinyl acetate	**12a**	66 % ee, 43 % yield	**12b** [9]
$PhCH_2$	Et	≥95 % ee, 39 % yield, PCL, propionic anhydride	**13a**	92 % ee, 43 % yield	**13b** [10]
1-Naphthyl	*n*-Pr	95 % ee, 47 % yield, PPL, $Cl_3CCH_2OCOnPr$	**14a**	≥95 % ee, 46 % yield	**14b** [11]
2-Naphthyl	Me	≥99 % ee, 41 % yield, PSL, vinyl acetate	**15a**	95 % ee, 48 % yield	**15b** [9]

Table 11.1-20. (cont.).



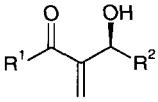
16a [9, 10]



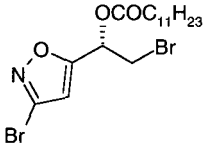
16b [9, 10]

≥95 % ee, 39 % yield, PCL, acetic anhydride

≥92 % ee, 43 % yield

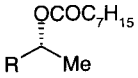


R^1	R^2		
n-BuO	Me	≥95 % ee, 41 % yield	**17** [12]
n-BuO	Pr	≥95 % ee, 23 % yield	**18** [12]
n-Bu	Me	≥95 % ee, 23 % yield	**19** [12]
$PhCH_2CH_2$	Me	≥95 % ee, 41 % yield all PCL, vinyl acetate	**20** [12]

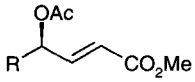


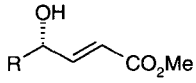
21 [13]

97 % ee, –, PSL, $CF_3CH_2OCOC_{11}H_{23}$

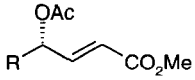


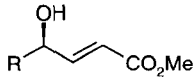
R				
n-C_6H_{13}	≥97 % ee, –	**22a**	≥98 % ee, –	**22b** [14]
Ph	97 % ee, –	**23a**	98 % ee, –	**23b** [14]
c-C_6H_{11}	95 % ee, –	**24a**	≥98 % ee, –	**24b** [14]
	all CAL-B, $C_7H_{15}COSEt$			





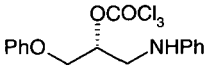
R				
Me	91 % ee, 39 % yield	**25a**	≥95 % ee, 38 % yield	**25b** [15]
Et	≥95 % ee, 45 % yield	**26a**	≥95 % ee, 44 % yield	**26b** [15]
n-Pr	74 % ee, 54 % yield	**27a**	≥95 % ee, 42 % yield	**27b** [15]
	all PSL, isopropenyl acetate			





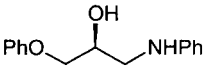
R				
i-Pr	19 % ee, 43 % yield	**28a**	≥95 % ee, 38 % yield	**28b** [16]
$Me_2ThexSi$-$O(CH_2)_2$	≥95 % ee, 35 % yield	**29a**	72 % ee, 57 % yield	**29b** [16]
	all PSL, isopropenyl acetate			

Table 11.1-20. (cont.).



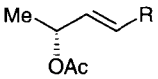
30a [17]

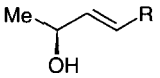
68 % ee, 26 % yield, PPL, $(CCl_3CO)_2O$



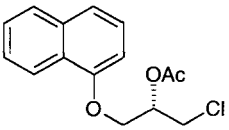
30b [17]

96 % ee, 28 % yield



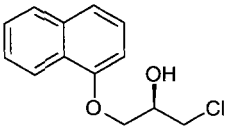


R					
$SiMe_3$	80 % ee, 34 % yield	**31a**	≥95 % ee, 38 % yield	**31b** [18]
$SiMe_2tBu$	≥95 % ee, 25 % yield	**32a**	≥95 % ee, 44 % yield	**32b** [18]
$SiMe_2Ph$	≥95 % ee, 30 % yield	**33a**	≥95 % ee, 42 % yield	**33b** [18]
	all PSL, vinyl acetate			



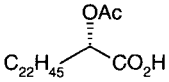
34a [19]

≥95 % ee, 45 % yield, PCL, vinyl acetate or acetic anhydride



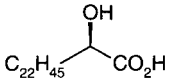
34b [19]

≥95 % ee, 47 % yield



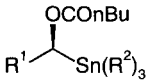
35a [20]

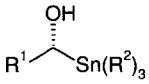
77 % ee, 55 % yield, PCL, vinyl acetate



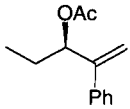
35b [20]

≥99 % ee, 45 % yield



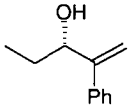


R^1	R^2				
Me	Me	97 % ee, 31 % yield	**36a**	71 % ee, 42 % yield	**36b** [21]
Et	Me	99 % ee, 36 % yield	**37a**	56 % ee, 36 % yield	**37b** [21]
n-Pr	Me	97 % ee, 7 % yield	**38a**	68 % ee, 7 % yield	**38b** [21]
Me	Et	99 % ee, 35 % yield	**39a**	51 % ee, 47 % yield	**39b** [21]
Et	Et	97 % ee, 14 % yield	**40a**	57 % ee, 14 % yield	**40b** [21]
		all PPL, n-$BuCO_2CH_2CF_3$			



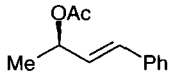
41a [22]

≥95 % ee, 47 % yield, PSL, vinyl acetate



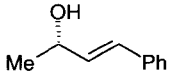
41b [22]

≥95 % ee, 32 % yield



42a [22]

≥95 % ee, 50 % yield, PSL, vinyl acetate



42b [22]

≥95 % ee, 47 % yield

Table 11.1-20. (cont.).

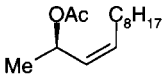
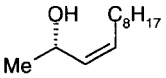
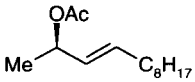
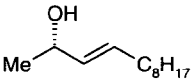
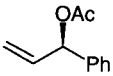
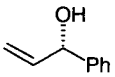
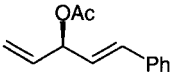
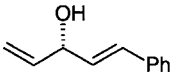
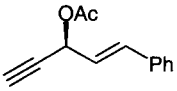
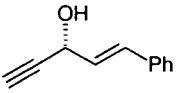
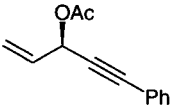
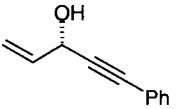
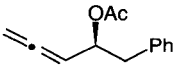
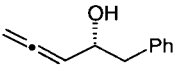
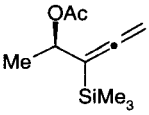
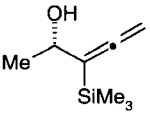
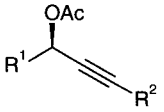
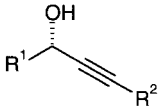
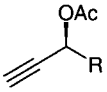
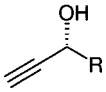
 31 % ee, 46 % yield, PSL, vinyl acetate	**43a** [22]	 56 % ee, 35 % yield	**43b** [22]
 33 % ee, 63 % yield, PSL, vinyl acetate	**44a** [22]	 ≥95 % ee, 22 % yield	**44b** [22]
 46 % ee, 32 % yield, PSL, vinyl acetate	**45a** [22]	 ≥95 % ee, 33 % yield	**45b** [22]
 ≥95 % ee, 49 % yield, PSL, vinyl acetate	**46a** [22]	 ≥95 % ee, 41 % yield	**46b** [22]
 68 % ee, 44 % yield, PSL, vinyl acetate	**47a** [22]	 ≥95 % ee, 28 % yield	**47b** [22]
 ≥95 % ee, 42 % yield, PSL, vinyl acetate	**48a** [22]	 ≥95 % ee, 43 % yield	**48b** [22]
 81 % ee, 47 % yield, PSL, vinyl acetate	**49a** [22]	 ≥95 % ee, 38 % yield	**49b** [22]
 ≥95 % ee, 38 % yield, PSL, vinyl acetate	**50a** [22]	 81 % ee, 31 % yield	**50b** [22]

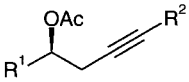
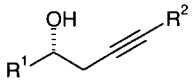
Table 11.1-20. (cont.).

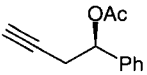
R^1	R^2				
Me	Ph	≥95 % ee, 48 % yield	**51a**	≥95 % ee, 47 % yield	**51b** [22]
Me	*n*-Bu	87 % ee, 41 % yield	**52a**	≥95 % ee, 31 % yield	**52b** [22]
Me	*n*-C_8H_{17}	≥95 % ee, 50 % yield	**53a**	≥95 % ee, 30 % yield	**53b** [22]
Me	$SiMe_3$	≥95 % ee, 45 % yield	**54a**	≥95 % ee, 27 % yield	**54b** [22]
Me	CH_2OCH_2-C_6H_4OMe	78 % ee, 48 % yield	**55a**	≥95 % ee, 46 % yield	**55b** [22]
Et	*n*-Bu	82 % ee, 36 % yield	**56a**	≥95 % ee, 46 % yield	**56b** [22]
C(Me)=CH_2	C(Me)=CH_2	≥95 % ee, 47 % yield all PSL, vinyl acetate	**57a**	≥95 % ee, 36 % yield	**57b** [22]

R					
CH_2Ph	77 % ee, 52 % yield	**58a**	95 % ee, 31 % yield	**58b** [22]
n-C_5H_{11}	–, 49 % yield all PSL, vinyl acetate	**59a**	23 % ee, 24 % yield	**59b** [22]

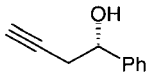



R^1	R^2				
Me	$SiMe_3$	≥95 % ee, 20 % yield	**60a**	≥95 % ee, 26 % yield	**60b** [22]
Et	Ph	50 % ee, 63 % yield	**61a**	≥95 % ee, 33 % yield	**61b** [22]
Me	Et	≥95 % ee, 44 % yield	**62a**	54 % ee, 21 % yield	**62b** [22]
Et	Et	38 % ee, 33 % yield all PSL, vinyl acetate	**63a**	61 % ee, 27 % yield	**63b** [22]



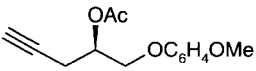
64a [22]

72 % ee, 54 % yield, PSL, vinyl acetate



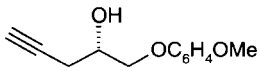
64b [22]

≥95 % ee, 34 % yield



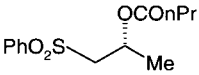
65a [23]

≥99 % ee, 47 % yield, PSL, vinyl acetate



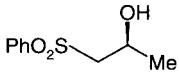
65b [23]

97 % ee, 49 % yield



66a [24]

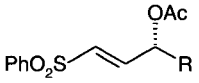
65 % ee, 20 % yield, PPL, CCl_3CH_2OCO*n*Pr

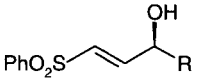


66b [24]

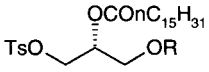
95 % ee, 25 % yield

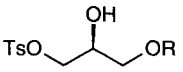
Table 11.1-20. (cont.).



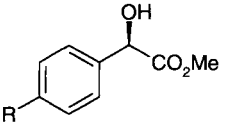


R				
Me	≥95 % ee, 47 % yield	**67a**	≥98 % ee, 46 % yield	**67b** [25, 26]
Et	≥95 % ee, 49 % yield	**68a**	≥98 % ee, 49 % yield	**68b** [25, 26]
i-Pr	≥95 % ee, 48 % yield	**69a**	94 % ee, 46 % yield	**69b** [25, 26]
n-C_6H_{13}	≥95 % ee, 47 % yield	**70a**	≥98 % ee, 48 % yield	**70b** [25, 26]
$PhCH_2O(CH_2)_2$	≥95 % ee, 47 % yield	**71a**	88 % ee, 48 % yield	**71b** [25, 26]
	all PPL, vinyl acetate			



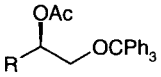


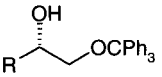
R				
n-$C_{16}H_{33}$	≥95 % ee, 45 % yield	**72a**	≥95 % ee, 43 % yield	**72b** [27]
n-$C_{10}H_{21}$	≥95 % ee, 43 % yield	**73a**	≥94 % ee, 42 % yield	**73b** [27]
n-Bu	≥95 % ee, 43 % yield	**74a**	≥96 % ee, 45 % yield	**74b** [27]
	all PSL, $(C_{15}H_{31}CO)_2O$			



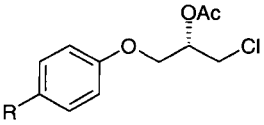
75 [28]

R = H, F, Cl, Br, OMe ≥99 % ee, –
all PSL, vinyl acetate

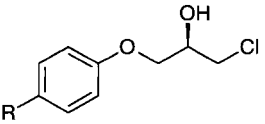




R				
$ClCH_2$	≥98 % ee, 43 % yield	**76a**	72 % ee, 54 % yield	**76b** [29]
Me	≥95 % ee, 37 % yield	**77a**	78 % ee, 40 % yield	**77b** [29]
Et	≥98 % ee, 43 % yield	**78a**	≥98 % ee, 48 % yield	**78b** [29]
n-Pr	70 % ee, 44 % yield	**79a**	56 % ee, 52 % yield	**79b** [29]
	all PCL, vinyl acetate			

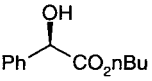


80a [30]



80b [30]

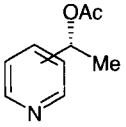
R = H, Me, OMe, Allyl, *c*-C_5H_{11}, CH_2CN, NO_2, O-Allyl
83–87 % ee, 36–53 % yield 86–96 % ee, 38–48 % yield
PSL, vinyl acetate



81 [31]

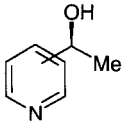
≥99 % ee, 43 % yield, PCL,
vinyl acetate

Table 11.1-20. (cont.).



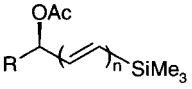
82a [32]

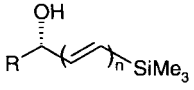
(2–4)
≥95 % ee, 41–50 % yield, PSL, vinyl acetate



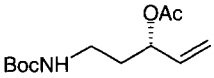
82b [32]

≥95 % ee, 33–38 % yield



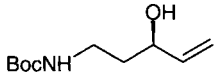


R	n				
Me	2	≥97 % ee, 23 % yield	**83a**	≥97 % ee, 38 % yield	**83b** [33]
n-C_5H_{11}	2	≥97 % ee, 40 % yield	**84a**	74 % ee, 33 % yield	**84b** [33]
Me	3	≥97 % ee, 41 % yield	**85a**	≥97 % ee, 34 % yield	**85b** [33]
n-C_5H_{11}	3	92 % ee, 15 % yield	**86a**	18 % ee, 56 % yield	**86b** [33]
Me	1	≥97 % ee, 32 % yield	**87a**	≥97 % ee, 30 % yield	**87b** [33]
n-C_5H_{11}	1	97 % ee, 47 % yield	**88a**	85 % ee, 267 % yield	**88b** [33]
		all PSL, vinyl acetate			



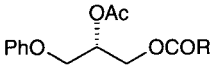
89a

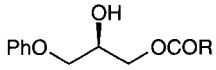
88 % ee, 49 % yield, PCL, vinyl acetate



89b [34]

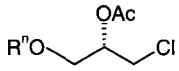
94 % ee, 43 % yield

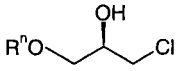


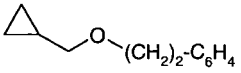


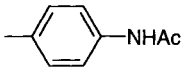
R					
Me	98 % ee, 49 % yield, PCL, vinyl acetate	**90a**	93 % ee, 51 % yield	**90b** [35]
n-C_7H_{15}	92 % ee, 45 % yield, PCL, vinyl acetate	**91a**	82 % ee, 49 % yield	**91b** [35]
n-$C_{15}H_{31}$	98 % ee, 48 % yield, PCL, vinyl acetate	**92a**	89 % ee, 52 % yield	**92b** [35]
i-Pr	96 % ee, 42 % yield, PCL, vinyl acetate	**93a**	52 % ee, 58 % yield	**93b** [35]
Ph	95 % ee, 17 % yield, PCL, vinyl acetate	**94a**	19 % ee, 78 % yield	**94b** [35]

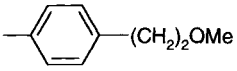
Table 11.1-20. (cont.).

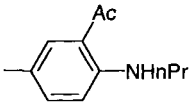




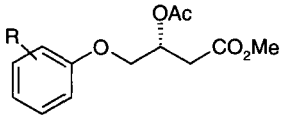
R^1 = 

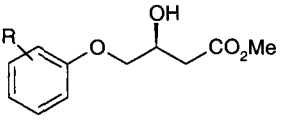
R^2 = Naphthyl R^3 = 

R^4 = 

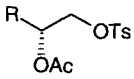
R^5 = 

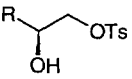
R^1	>98 % ee, 45 % yield, PSL, vinyl acetate	**95a**	91 % ee, 50 % yield	**95b** [36]
R^2	95 % ee, –, CAL-B, vinyl acetate	**96a**	27 % ee, –	**96b** [37]
R^3	95 % ee, –, CAL-B, vinyl acetate	**97a**	18 % ee, –	**97b** [37]
R^4	95 % ee, –, CAL-B, vinyl acetate	**98a**	20 % ee, –	**98b** [37]
R^5	95 % ee, –, CAL-B, vinyl acetate	**99a**	28 % ee, –	**99b** [37]



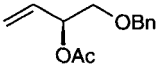


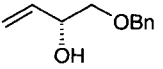
R				
4-OMe	99 ee, –, PCL, vinyl acetate, 49 % conversion	**100a**	99 ee, – 99 ee, –	**100b** [38]
2-Allyl	99 ee, –, PCL, vinyl acetate, 49 % conversion	**101a**	49 ee, –	**101b** [38]
2,3-C_4H_4	99 ee, –, PCL, vinyl acetate, 33 % conversion	**102a**		**102b** [38]





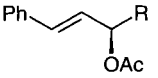
R				
$CH{=}CH_2$	96 % ee, –, PCL, vinyl acetate	**103a**	98 % ee, –	**103b** [39]
	95 % ee, 48 % yield, PCL,	***ent*-103a**	84 % ee, 49 % yield	***ent*-103b** [40]
Me	vinyl acetate			**104b** [39]
CH_2Cl	93 % ee, –, PCL, vinyl acetate	**104a**	99 % ee, –	**105b** [39]
Et	92 % ee, –, PCL, vinyl acetate	**105a**	>99 % ee, –	**106b** [39]
	80 % ee, –, PCL, vinyl acetate	**106a**	98 % ee, –	

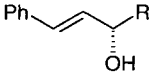




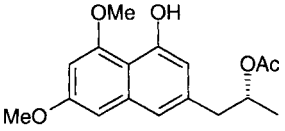
79 % ee,45 % yield, PCL, vinyl acetate	**107a**	70 % ee, 55 % yield	**107b** [40]

Table 11.1-20. (cont.).

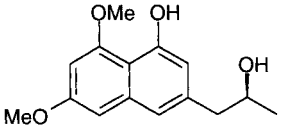




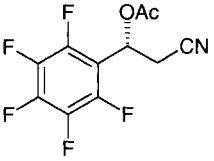
R				
CH_2OBz	98 % ee, 43 % yield, PCL, vinyl acetate	**108a**	88 % ee, 49 % yield	**108b** [41]
CO_2Me	98 % ee, 47 % yield, PCL, vinyl acetate	**109a**	98 % ee, 47 % yield	**109b** [41]



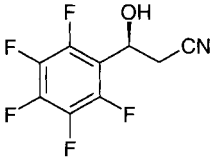
110a [42]
>99 % ee, 42 % yield, PFL, vinyl acetate



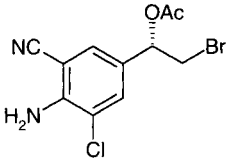
110b [42]
72 % ee, 58 % yield



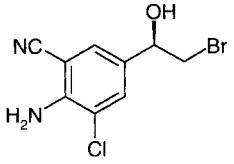
111a [43]
>99 % ee, 45 % yield, LIP, vinyl acetate



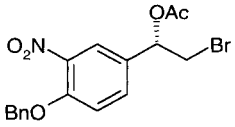
111b [43]
>99 % ee, 42 % yield



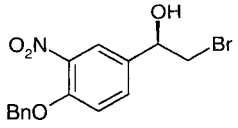
112a [44]
90 % ee, 46 % yield, PCL, vinyl acetate



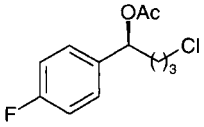
112b [44]
97 % ee, 44 % yield



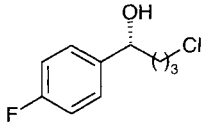
113a [45]
86 % ee, 48 % yield, PCL, vinyl acetate



113b [45]
96 % ee, 46 % yield

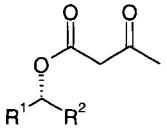


114a [46]
93 % ee, –, PCL, isopropenyl acetate, 46 % conversion



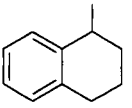
114b [46]
90 % ee, –

Table 11.1-20. (cont.).



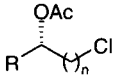
115a [47]

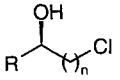
115b [47]



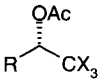
R^1 = Ph, 1-naphthyl, 2-naphthyl, benzyl, *n*-hexyl, R^2 = Me; R^1, R^2 = 50–99 % ee, 39–48 % yield, PCL, PSL, CAL-B, diketene

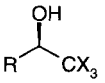
95–99 % ee, 30–43 % yield



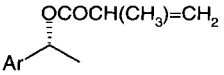


R	n				
Ph	2	92 % ee, 49 % yield, PCL, isopropenyl acetate	**116a**	99 % ee, 44 % yield	**116b** [48]
Ph	2	97 % ee, 31 % yield, CAL-B, vinyl butanoate	**116a**	96 % ee, 33 % yield	**116b** [49]
4-*t*-Bu-C_6H_4	3	99 % ee, 47 % yield, PCL, isopropenyl acetate	**117a**	99 % ee, 47 % yield	**117b** [48]
4-*t*-Bu-C_6H_4	3	>95 % ee, –, PCL, vinyl acetate, 50 % conversion	**117a**	>95 % ee, –	**117b** [50]
1-o-Naphthyl	1	89 % ee, 41 % yield, PCL, isopropenyl acetate	**118a**	99 % ee, 44 % yield	**118b** [48]
Ph	3	79 % ee, –, PCL, vinyl acetate, 55 % conversion	**119a**	>95 % ee, –	**119b** [50]
4-F-C_6H_4	3	>95 % ee, –, PCL, vinyl acetate, 50 % conversion	**120a**	>95 % ee, –	**120b** [50]
4-F-C_6H_4	3	97 % ee, 44 % yield, PCL, vinyl acetate	**120a**	85 % ee, 48 % yield	**120b** [51]





R	X				
2-Naphthyl	F	85 % ee, 37 % yield, PCL, vinyl acetate	**121a**	>99 % ee, 51 % yield	**121b** [52]
2-Naphthyl	H	97 % ee, 37 % yield, PCL, vinyl acetate	**122a**	99 % ee, 43 % yield	**122b** [52]
1-Naphthyl	H	>99 % ee, 32 % yield, PCL, vinyl acetate	**123a**	69 % ee, 40 % yield	**123b** [52]



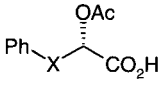
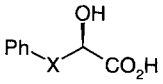
124a [53]

124b [53]

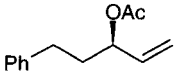
85–97 % ee, –, PCL, 2,3-butanedione monooxime methacrylate, 43–47 % conversion

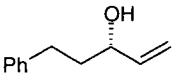
87–95 % ee, –

Table 11.1-20. (cont.).

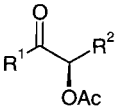
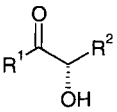
X				
$(CH_2)_2$	84 % ee, 35 % yield, PCL, vinyl acetate	**125a**	>99 % ee, 45 % yield	**125b** [54]
(*E*)-CH=CH	94 % ee, 34 % yield, PCL, vinyl acetate	**126a**	>99 % ee, 42 % yield	**126b** [54]


127a [55]

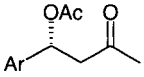

127b [55]

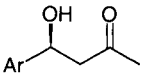
98 % ee, 48 % yield, PCL, vinyl acetate — 92 % ee, 48 % yield

>99 % ee, 49 % yield, PCL, vinyl acetate, 1,4,8,11-tetrathiacyclo-tetradecane as additive — 98 % ee, 51 % yield

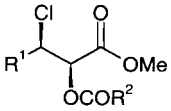
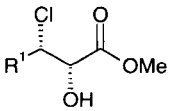



R^1	R^2				
Ph	Me	95 % ee, –, PSL, isopropenyl acetate, 48 % conversion	**128a**	89 % ee, –	**128b** [56]
		99 % ee, –, PCL, isopropenyl acetate, 19 % conversion		89 % ee, –	
Me	Ph	98 % ee, –, PSL, isopropenyl acetate, 48 % conversion	**129a**	92 % ee, –	**129b** [56]
		98 % ee, –, PCL, isopropenyl acetate, 46 % conversion		83 % ee, –	


130a [57]

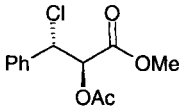

130b [57]

>96 % ee, 26–44 % yield, CCL, vinyl acetate — 33–70 % ee, 55–73 % yield

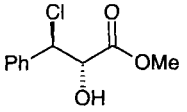



R^1	R^2				
Ph	Me	93 % ee, 45 % yield, PCL, vinyl acetate	**131a**	95 % ee, 50 % yield	**131b** [58]
Ph	Ph	>99 % ee, 12 % yield, lipase SL (Meito), vinyl benzoate	**132a**	61 % ee, 41 % yield	**131b** [58]
Alkyl	Me	30–98 % ee, 42–74 % yield, PCL, vinyl acetate	**133a**	53–99 % ee, 17–43 % yield	**133b** [59]

Table 11.1-20. (cont.).



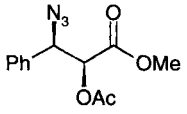
134a [58]



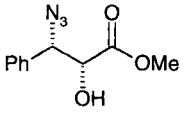
134b [58]

93 % ee, 45 % yield, PCL, vinyl acetate
97 % ee, 31 % yield, lipase SL (Meito), vinyl acetate

>99 % ee, 46 % yield
42 % ee, 46 % yield



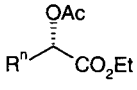
135a [58]

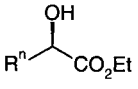


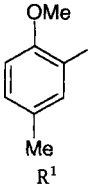
135b [58]

76 % ee, 38 % yield, PCL, vinyl acetate

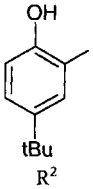
80 % ee, 44 % yield



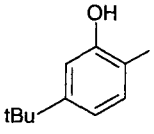




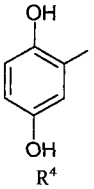
R^1



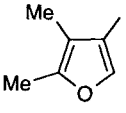
R^2



R^3

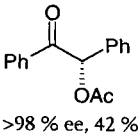


R^4

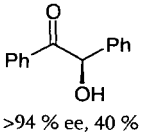


R^5

R^1	97 % ee, 46 % yield, PCL (PS-C), vinyl acetate, 48 % conversion	**136a**	–, –	**136b** [60]
R^2	97 % ee, 41 % yield, CRL, vinyl acetate, 45 % conversion	**137a**	–, –	**137b** [60]
R^3	95 % ee, 45 % yield, PCL (PS-C), vinyl acetate, 47 % conversion	**138a**	–, –	**138b** [60]
R^4	97 % ee, 48 % yield, CRL, vinyl acetate, 51 % conversion	**139a**	–, –	**139b** [60]
R^5	97 % ee, 40 % yield, PCL (PS-C), vinyl acetate, 46 % conversion	**140a**	–, –	**140b** [60]



141a [61]

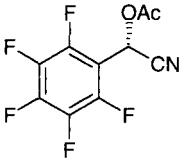


141b [61]

>98 % ee, 42 % yield, lipase TL, vinyl acetate

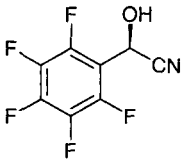
>94 % ee, 40 % yield

Table 11.1-20. (cont.).



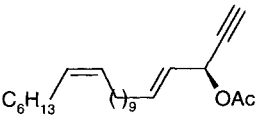
142a [62]

98 % ee, 50 % yield, LIP, vinyl acetate



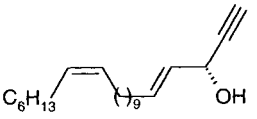
142b [62]

96 % ee, 46 % yield



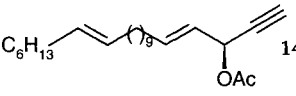
143a [63]

95 % ee, 45 % yield, CAL-B, vinyl acetate



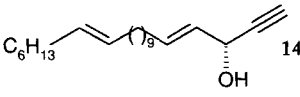
143b [63]

65 % ee, 49 % yield



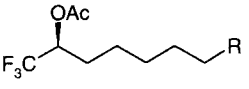
144a [63]

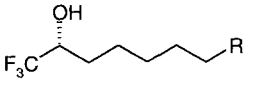
94 % ee, 42 % yield, CAL-B, vinyl acetate



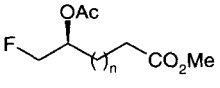
144b [63]

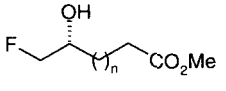
81 % ee, 51 % yield





R				
Me	>99 % ee, –, CAL-B, vinyl acetate, 25 % conversion	**145a**	33 % ee, –	**145b** [64]
Et	97 % ee, –, CAL-B, vinyl acetate, 35 % conversion	**146a**	52 % ee, –	**146b** [64]
n-Pr	>99 % ee, –, CAL-B, vinyl acetate, 25 % conversion	**147a**	33 % ee, –	**147b** [64]
n-Bu	>99 % ee, –, CAL-B, vinyl acetate, 32 % conversion	**148a**	12 % ee, –	**148b** [64]
CH_2OPh	>99 % ee, –, CAL-B, vinyl acetate, 14 % conversion	**149a**	25 % ee, –	**149b** [64]

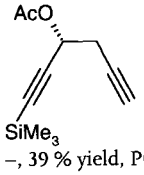




n				
6	94 % ee, 48 % yield, PCL, acetic anhydride	**150a**	66 % ee, 35 % yield	**150b** [65]
7	90 % ee, 38 % yield, PCL, acetic anhydride	**151a**	54 % ee, 44 % yield	**151b** [65]

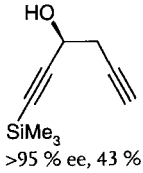
Table 11.1-20. (cont.).

R^1	R^2				
n-C_6H_{13}	Me	97 % ee, –, CAL-B, n-$C_7H_{15}COSEt$, 50 % conversion	**152a**	98 % ee, –	**152b** [66]
n-C_8H_{17}	C≡CH	95 % ee, –, CAL-B, n-$C_7H_{15}COSEt$, 50 % conversion	**153a**	96 % ee, –	**153b** [66]
n-C_6H_{13}	CH=CH$_2$	96 % ee, –, CAL-B, n-$C_7H_{15}COSEt$, 49 % conversion	**154a**	93 % ee, –	**154b** [66]
n-C_6H_{13}	ET	96 % ee, –, CAL-B, n-$C_7H_{15}COSEt$, 51 % conversion	**155a**	>99 % ee, –	**155b** [66]



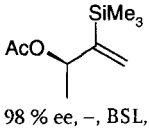
156a [67]

–, 39 % yield, PCL,vinyl acetate



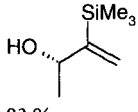
156b [67]

>95 % ee, 43 % yield



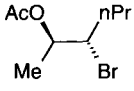
157a [68]

98 % ee, –, BSL, vinyl acetate, 49 % conversion



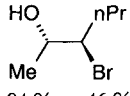
157b [68]

93 % ee, –,



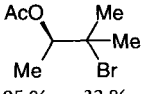
158a [69]

94 % ee, 30 % yield, PSL, vinyl acetate



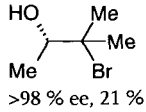
158b [69]

84 % ee, 46 % yield



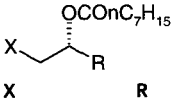
159a [69]

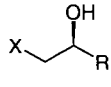
95 % ee, 32 % yield, PSL, vinyl acetate



159b [69]

>98 % ee, 21 % yield





X	R				
Cl	Me	97 % ee, –, CAL-B, n-$C_7H_{15}COSEt$, 42 % conversion	**160a**	71 % ee, –	**160b** [70]
Br	Me	98 % ee, –, CAL-B, n-$C_7H_{15}COSEt$, 47 % conversion	**161a**	88 % ee, –	**161b** [70]
Br	Et	96 % ee, –, CAL-B, n-$C_7H_{15}COSEt$, 30 % conversion	**162a**	41 % ee, –	**162b** [70]

Table 11.1-20. (cont.).

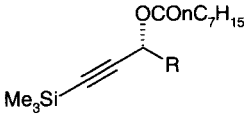
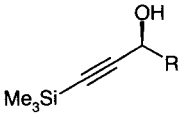
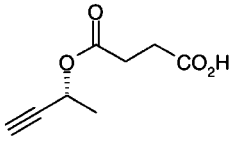
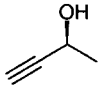
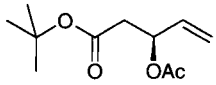
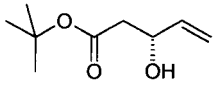
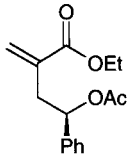
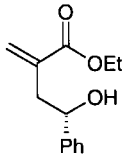
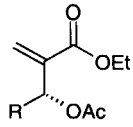
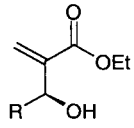
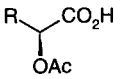
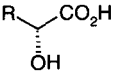
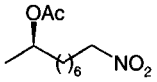
				
R				
Me	99 % ee, –, CAL-B, n-$C_7H_{15}COSEt$	**163a**	95 % ee, –	**163b** [70]
Et – n-$C_{13}H_{27}$	81–96 % ee, 24–49 % yield, PCL, vinyl acetate	**164a**	36–>98 % ee, 41–71 % yield	**164b** [71]
	**165a** [72]			**165b** [72]
71 % ee, –, PSL, succinic anhydride, 58 % conversion		98 % ee, –		
	**166a** [73]			**166b** [73]
>99 % ee, 45 % yield, PCL, vinyl acetate		–, –		
	167a [74]			**167b** [74]
>98 % ee, –, CAL-B, isopropenyl acetate, 49 % conversion		94 % ee, –		
	**168a** [75]			**168b** [75]
99 % ee, 35 % yield, BSL, vinyl acetate		99 % ee, 36 % yield		
				
R				
Me	87 % ee, 39 % yield, BSL, vinyl acetate	**169a**	99 % ee, 35 % yield	**169b** [76]
Et	98 % ee, 38 % yield, BSL, vinyl acetate	**170a**	98 % ee, 42 % yield	**170b** [76]

Table 11.1-20. (cont.).



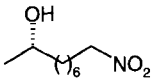


R				
$(CH_2)_{13}Me$	98 % ee, –, BSL, vinyl acetate, 48 % conversion	**171a**	91 % ee, –	**171b** [77]
CH_2Ph	98 % ee, –, BSL, vinyl acetate, 48 % conversion	**172a**	89 % ee, –	**172b** [77]



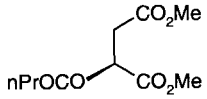
91 % ee, 31 % yield, GLL, vinyl acetate

173a [78]



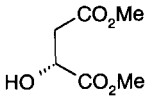
–, –

173b [78]



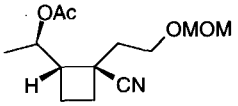
82 % ee, 49 % yield, CAL-A, vinyl butanoate

174a [79]



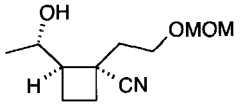
96 % ee, 35 % yield

174b [79]



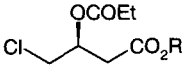
>99 % ee, 35–44 % yield, PCL, CAL-B, PFL, vinyl acetate

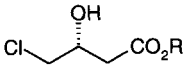
175a [80]



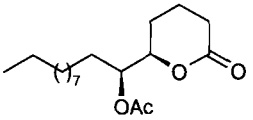
73–99 % ee, 47–56 % yield

175b [80]





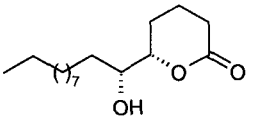
R				
Et	96 % ee, 29 % yield, RML, vinyl propionate	**176a**	–, –	**176b** [81]
CH_2Ph	77 % ee, 43 % yield, RML, vinyl propionate	**177a**	96 % ee, 29 % yield	**177b** [81]
c-C_6H_{11}	89 % ee, 45 % yield, RML, vinyl propionate	**178a**	96 % ee, 44 % yield	**178b** [81]
t-Bu	>97 % ee, 48 % yield, RML, vinyl propionate	**179a**	99 % ee, 42 % yield	**179b** [81]



95 % ee, –, CAL-A, vinyl acetate, 25 % conversion, 55 °C

83 % ee, –, CAL-A, vinyl acetate, 56 % conversion, 22 °C

180a [82]

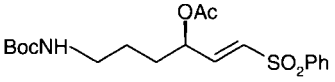


32 % ee, –

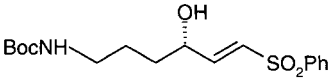
>99 % ee, –

180b [82]

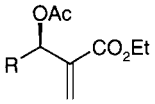
Table 11.1-20. (cont.).

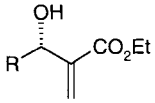


181a [83]
>99 % ee, 49 % yield, PCL, vinyl acetate

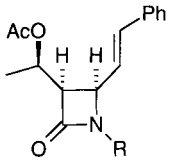


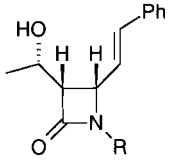
181b [83]
>99 % ee, 46 % yield



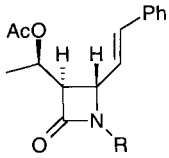


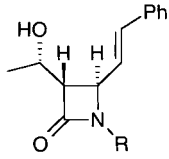
R				
Me	97 % ee, 43 % yield, PCL, isopropenyl acetate	**182a**	>97 % ee, 42 % yield	**182b** [84]
Et	>99 % ee, 37 % yield, PCL, vinyl acetate	**183a**	70 % ee, 50 % yield	**183b** [84]



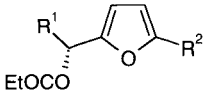


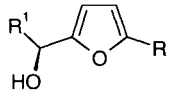
R				
H	>97 % ee, 24 % yield, PCL, vinyl acetate	**184a**	41 % ee, 69 % yield	**184b** [85]
PMP	>97 % ee, 29 % yield, PCL, vinyl acetate	**185a**	44 % ee, 61 % yield	**185b** [85]





R				
H	>97 % ee, 49 % yield, PCL, vinyl acetate	**186a**	>97 % ee, 47 % yield	**186b** [85]
TBDMS	>97 % ee, 42 % yield, PCL, vinyl acetate	**187a**	>97 % ee, 49 % yield	**187b** [85]





R^1	R^2			
CF_3	TMS	>99 % ee, –, CAL-B, vinyl propionate, 42 % conversion	**188a**	72 % ee, –	**188b** [86]
CHF_2	TBDMS	>99 % ee, –, CAL-B, vinyl propionate, 36 % conversion	**189a**	53 % ee, –	**189b** [86]
C_2F_5	TMS	98 % ee, –, LIP,	**190a**	22 % ee, –	**190b** [86]

Table 11.1-20. (cont.).

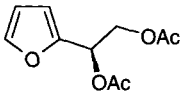
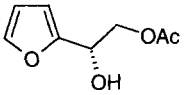
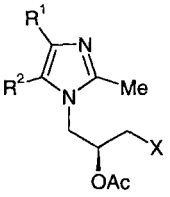
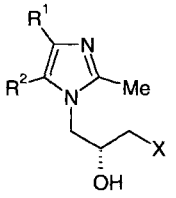
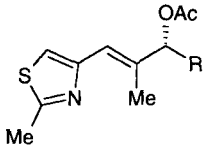
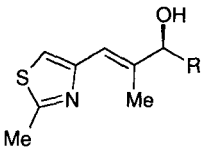
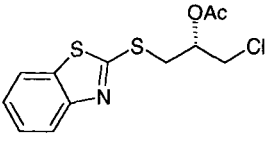
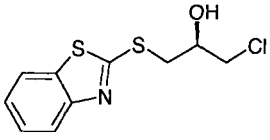
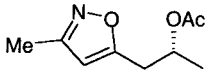
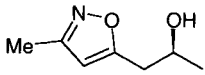
 82 % ee, 52 % yield, PCL, vinyl acetate	**191a** [87]	 >99 % ee, 47 % yield	**191b** [87]
			
R^1 = H, R^2 = NO_2, X = Cl 95 % ee, 41 % yield, PCL, vinyl acetate	**192a**	89 % ee, 43 % yield	**192b** [88]
R^1 = H, R^2 = NO_2, X = F >98 % ee, 41 % yield, PCL, vinyl acetate	**193a**	95 % ee, 43 % yield	**193b** [88]
R^1 = H, R^2 = NO_2, X = Br 94 % ee, 21 % yield, PCL, vinyl acetate	**194a**	39 % ee, 45 % yield	**194b** [88]
R^1 = NO_2, R^2 = H, X = Cl 92 % ee, 36 % yield, PCL, vinyl acetate	**195a**	95 % ee, 43 % yield	**195b** [88]
R^1 = NO_2, R^2 = H, X = F 96 % ee, 34 % yield, PCL, vinyl acetate	**196a**	48 % ee, 45 % yield	**196b** [88]
			
R			
$CH_2CH{=}CH_2$ –, –, PSL, vinyl acetate	**197a**	88 % ee, 46 % yield	**197b** [89]
$CH_2C{\equiv}CH$ –, –, PSL, vinyl acetate	**198a**	94 % ee, 40 % yield	**198b** [89]
$CH{=}CH_2$ –, –, PSL, vinyl acetate	**199a**	90 % ee, 48 % yield	**199b** [89]
 93 % ee, –, PFL, vinyl acetate, 31 % conversion	**200a** [90]	 42 % ee, –	**200b** [90]

Table 11.1-20. (cont.).



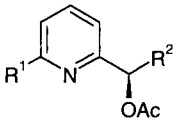
201a [91]

89 % ee, 36 % yield, CAL-B, vinyl acetate

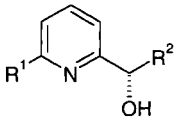


201b [91]

62 % ee, 23 % yield

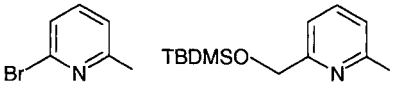


202a [92]



202b [92]

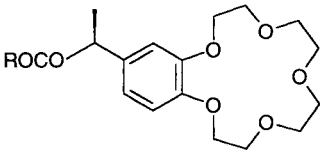
R^1 = H, Br, Me, TBDMS-OCH_2, $TrOCH_2$, Ph, 2-Py

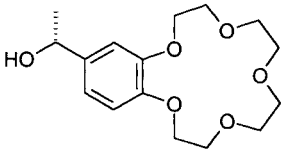


R^2 = Me, Et, Vinyl

92–99 % ee, 31–49 % yield, CAL-B, vinyl acetate

78–99 % ee, 43–58 % yield

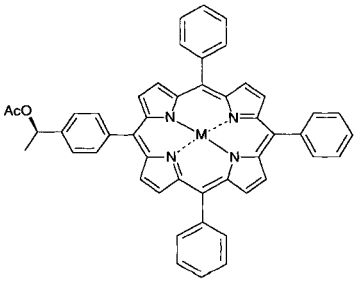


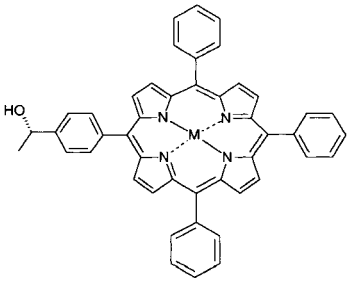


R = Me, *n*-Pr, *n*-C_5H_{11}, *n*-C_7H_{15}

98–>99 % ee, 22–48 % yield, PCL or CAL-B, vinyl alkanoate **203a** [93]

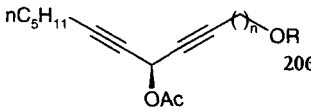
35–96 % ee, 25–45 % yield **203b** [93]





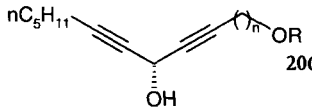
M				
H_2	>98 % ee, 44–46 % yield, PCL, CAL-B or LIP, vinyl acetate	**204a**	89–>98 % ee, 36–47 % yield	**204b** [94]
Zn	>98 % ee, 12–24 % yield, PCL, CAL-B or LIP, vinyl acetate	**205a**	16–>95 % ee, 72–82 % yield	**205b** [94]

Table 11.1-20. (cont.).



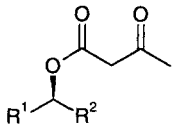
206a [95]

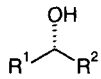
n = 1–3, TBDMS, TBDPS
94–95 % ee, 35–42 % ee, CRL, vinyl acetate



206b [95]

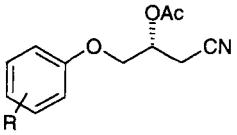
58–77 % ee, 33–46 % ee





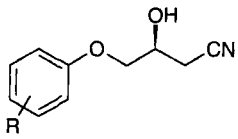
R^1	R^2				
Ph	Me	96 % ee, 41 % yield	**207a**	98 % ee, 45 % yield	**207b** [96]
$Ph(CH_2)_2$	Vinyl	92 % ee, 42 % yield	**208a**	96 % ee, 44 % yield	**208b** [96]
PhCH=CH	Me	97 % ee, 41 % yield	**209a**	90 % ee, 38 % yield	**209b** [96]
2-Naphthyl	Me	93 % ee, 40 % yield	**210a**	96 % ee, 46 % yield	**210b** [96]

all CAL-B, methyl acetoacetate



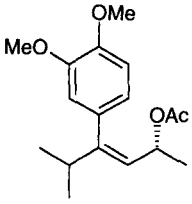
211a [97]

R = H, 2-Me, 4-Me, 4-Cl, 4-Br, 2,3-C_4H_4, 3,4-C_4H_4
89- >99 % ee, 34–55 % yield, PCL, vinyl acetate



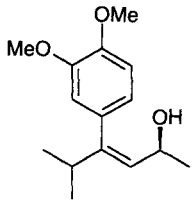
211b [97]

35–94 % ee, 41–62 % yield



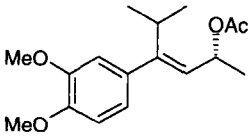
212a [98]

92 % ee, 30 % yield, PCL, vinyl acetate



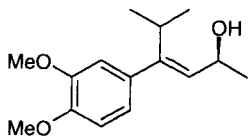
212b [98]

47 % ee, 68 % yield



213a [98]

92 % ee, 38 % yield, PCL, vinyl acetate



213b [98]

76 % ee, 51 % yield

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96 A. Córdova, K. D. Janda, *J. Org. Chem.* **2001**, *66*, 1906.
97 A. Kamal, G. B. R. Khanna, *Tetrahedron: Asymmetry* **2001**, *12*, 405.
98 E. Brenna, C. Fuganti, P. Grasselli, S. Serra, *Eur. J. Org. Chem.* **2001**, 1349.

Monoacetates and alcohols of Table 11.1-19 which can be obtained with other hydrolases as such or of opposite configuration are contained in Tables 11.1-6 and 11.1-14.

For a wide structural range of racemic secondary alcohols, lipase-catalyzed enantiomer-differentiating acylation has been reported (**1–213**) (Table 11.1-20). The results show that this is a general method for the attainment of enantiomerically pure secondary alcohols that is complementary to the lipase-catalyzed hydrolysis of the corresponding acylated alcohols (Table 11.1-15). It is especially worth mentioning that secondary alcohols of the alkyl-alkyl, alkyl-aryl or alkyl-heteroaryl type, but also those bearing the various functional groups including stannylated derivatives, are accessible too. Acylation has been utilized in depth for the synthesis of allylic, homoallylic, propargylic and homopropargylic and allenylic alcohols (**17–20**, **25–27**,

Table 11.1-21. Lipase-catalyzed enantiomer-differentiating acylation of racemic cyclic secondary alcohols in organic solvents (CCL *Candida cylindracea* lipase, PSL *Pseudomonas* sp. lipase, CAL-B *Candida antarctica* B lipase, PPL pig pancreas lipase, PCL *Pseudomonas cepacia* lipase, LIP *Pseudomonas* sp. lipase-Toyobo, ASL *Alcaligenes* sp. lipase, PFL *Pseudomonas fluorescens* lipase, BSL *Burkholderia* sp. lipase, CRL *Candida rugosa* lipase, MML *Mucor miehei* lipase).

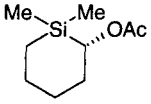
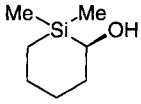
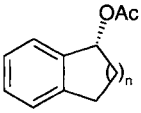
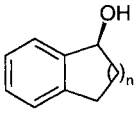
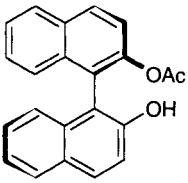
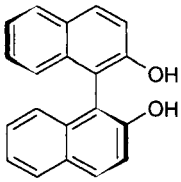
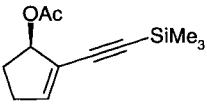
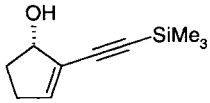
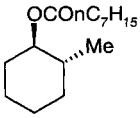
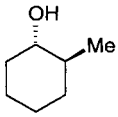
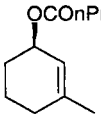
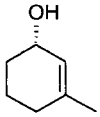
	**1a** [1]		**1b** [1]
95 % ee, 48 % yield, CCL, triacetin		96 % ee, 48 % yield	
			
n			
1		≥99 % ee, 46 % yield	**2a**	95 % ee, 48 % yield	**2b** [2]
2		97 % ee, 49 % yield, all PSL, vinyl acetate	**3a**	≥99 % ee, 44 % yield	**3b** [2]
	**4a** [3]		**4b** [3]
95 % ee, 52 % yield, PSL, vinyl acetate		89 % ee, 48 % yield	
	**5a** [4]		**5b** [4]
≥99 % ee, 48 % yield, PSL, vinyl acetate		≥99 % ee, 47 % yield	
	**6a** [5]		**6b** [5]
97 % ee, –, CAL-B, n-$C_7H_{15}COSEt$		97 % ee, –	
	**7a** [6]		**7b** [6]
87 % ee, – 95 % ee, –, (triple resolution) PSL, vinyl acetate		98 % ee, –	

Table 11.1-21. (cont.).

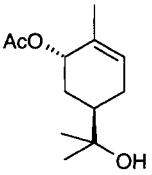
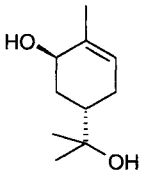
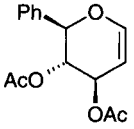
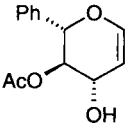
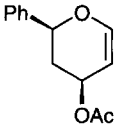
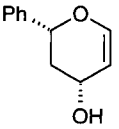
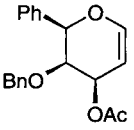
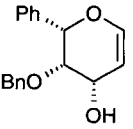
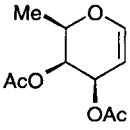
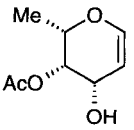
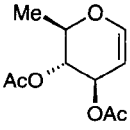
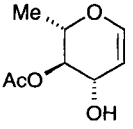
	**8a** [7]		**8b** [7]
95 % ee, 51 % yield, PCL, vinyl acetate		≥998 % ee, 39 % yield	
	**9a** [8, 9]		**9b** [8, 9]
≥97 % ee, 47 % yield, PCL, vinyl acetate		≥97 % ee, 47 % yield	
	**10a** [8, 9]		**10b** [8, 9]
60 % ee, 63 % yield, PCL, vinyl acetate		≥97 % ee, 37 % yield	
	**11a** [8, 9]		**11b** [8, 9]
91 % ee, 50 % yield, PCL, vinyl acetate		≥97 % ee, 48 % yield	
	**12a** [8, 9]		**12b** [8, 9]
≥97 % ee, 37 % yield, PCL, vinyl acetate		64 % ee, 51 % yield	
	**13a** [8, 9]		**13b** [8, 9]
≥97 % ee, 48 % yield, PCL, vinyl acetate		≥97 % ee, 48 % yield	

Table 11.1-21. (cont.).

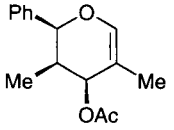
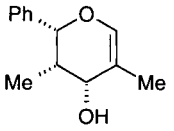
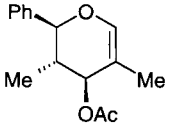
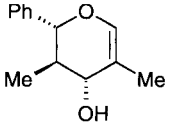
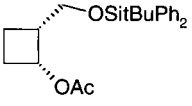
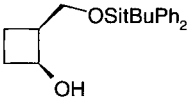
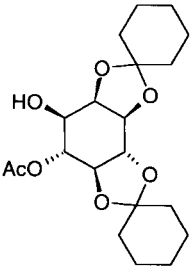
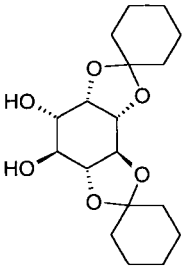
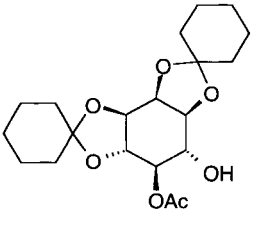
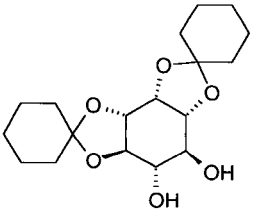
	**14a** [8, 9]		**14b** [8, 9]
70 % ee, 59 % yield, PCL, vinyl acetate		≥97 % ee, 41 % yield	
	**15a** [8, 9]		**15b** [8, 9]
≥96 % ee, 26 % yield, PCL, vinyl acetate		≥97 % ee, 44 % yield	
	**16a** [10]		**16b** [10]
90 % ee, 50 % yield, PCL, vinyl acetate		98 % ee, 50 % yield	
	**17a** [11]		**17b** [11]
100 % ee, 48 % yield, CCL, acetic anhydride		98 % ee, 51 % yield	
	**18a** [11]		**18b** [11]
96 % ee, 50 % yield, CCL, acetic anhydride		100 % ee, 48 % yield	

Table 11.1-21. (cont.).

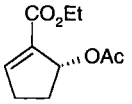
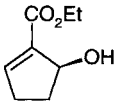
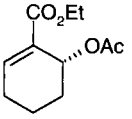
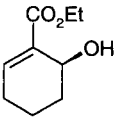
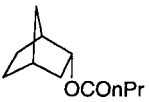
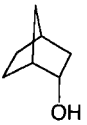
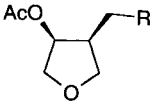
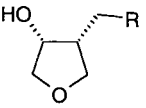
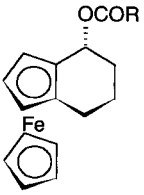
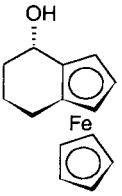
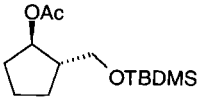
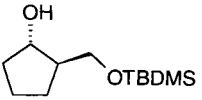
	**19a** [12, 13]		**19b** [12, 13]
≥98 % ee, 48 % yield, PCL, vinyl acetate		≥98 % ee, 48 % yield	
	**20a** [13]		**20b** [13]
99 % ee, 40 % yield, PCL, vinyl acetate		95 % ee, 53 % yield	
	**21a** [14]		**21b** [14]
87 % ee, –, PPL, n-$PrCO_2CH_2CCl_3$		87 % ee, –	
	**22a** [15]		**22b** [15]
R = Ph, 3-$MeOC_6H_4$, 3,4-$(MeO)_2C_6H_3$, 3,4-(methylenedioxy)C_6H_3			
≥99 % ee, –, PCL, vinyl acetate		≥99 % ee, –	
			
R			
Me	99 % ee, 38 % yield	**23a**	71 % ee, 51 % yield	**23b** [16]
n-Pr	99 % ee, 47 % yield	**24a**	94 % ee, 48 % yield	**24b** [16]
	PCL, vinyl acetate or butyric anhydride			
	**25a** [17]		**25b** [17]
94 % ee, 38 % yield, PCL, vinyl acetate		51 % ee, 57 % yield	

Table 11.1-21. (cont.).

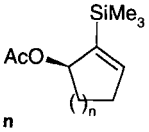
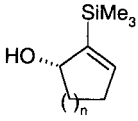
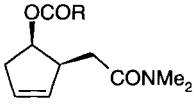
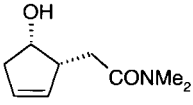
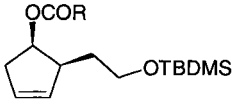
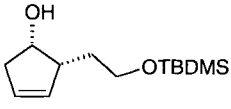
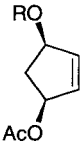
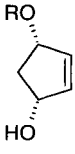
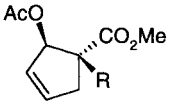
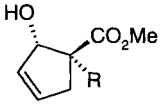
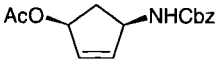
				
n				
1	93 % ee, –, PCL, vinyl acetate, 50 conversion	**26a**	95 % ee, –	**26b** [18]
	97 % ee, –, BSL, vinyl acetate, 50 conversion		>99 % ee, –	
2	99 % ee, –, BSL, vinyl acetate, 50 conversion	**27a**	98 % ee, –	**27b** [18]
		**28a** [19]		**28b** [19]
R = Me, Et, *n*-Pr, *n*-C_5H_{11}, *n*-C_7H_{15}, *n*-C_9H_{19}, $ClCH_2$				
70–97 % ee, 41–51 % yield, PCL, vinyl alkanoate			69–95 5 ee, 40–51 % yield	
		**29a** [19]		**29b** [19]
R = Me, *n*-Pr, *n*-C_9H_{19}, $ClCH_2$, Ph				
98– >99 % ee, 40–46 % yield, PCL, vinyl alkanoate			67– >99 % ee, 50–58 % yield	
				
R				
Ph	>95 % ee, 45 % yield, PCL, isopropenyl acetate	**30a**	>95 % ee, 49 % yield	**30a** [20]
PMP	87–90 % ee, 40 % yield, PCL, isopropenyl acetate	**31a**	–, 41 % yield	**31b** [20]
t-Bu	97 % ee, 48 % yield, PCL, vinyl acetate	**32a**	91 % ee, 53 % yield	**32b** [21]
CMe_2Ph	76 % ee, 51 % yield, pancreatin, vinyl acetate	**32a**	98 % ee, 40 % yield	**32b** [23b]
TBDMS	>99 % ee, 43 % yield, PCL, vinyl acetate	**33a**	>99 % ee, 50 % yield	**33b** [22]
Bn	98 % ee, 48 % yield, pancreatin, vinyl acetate	**34a**	98 % ee, 47 % yield	**34b** [23]
THP	30 % ee, 74 % yield, pancreatin, vinyl acetate	**35a**	98 % ee, 20 % yield	**35b** [23b]
	91 % ee, 45 % yield, pancreatin, vinyl acetate	**36a**	94 % ee, 50 % yield	**36b** [23b]

Table 11.1-21. (cont.).





R				
Me	91 % ee, 30 % yield, PSL, vinyl acetate	**37a**	63 % ee, 59 % yield	**37b** [24]
n-C_9H_{19}	96 % ee, 20 % yield, PSL, vinyl acetate	**38a**	40 % ee, 75 % yield	**38b** [24]
CH_2Ph	91 % ee, 21 % yield, PSL, vinyl acetate	**39a**	43 % ee, 63 % yield	**39b** [24]

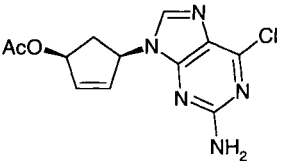


40a [25]

92 % ee, 40 % yield, PSL, vinyl acetate

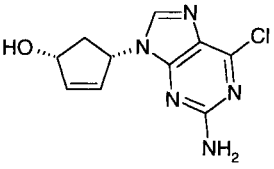
40b [25]

–, –



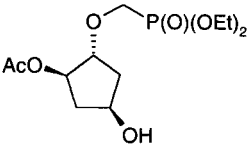
41a [26]

>90 % ee, 35 % yield, PCL, vinyl acetate



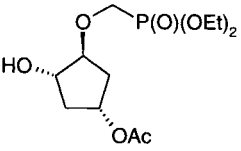
41b [26]

>90 % ee, 33 % yield



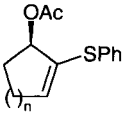
42a [27]

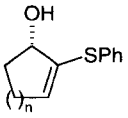
72 % ee, 42 % yield, PCL, vinyl acetate



42b [27]

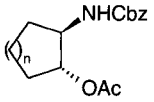
95 % ee, 40 % yield

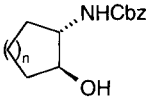




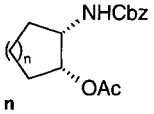
n				
1	93 % ee, 50 % yield, PCL, vinyl acetate	**43a**	100 % ee, 47 % yield	**43b** [28]
2	98 % ee, 48 % yield, PCL, vinyl acetate	**44a**	100 % ee, 47 % yield	**44b** [28]

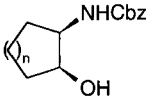
Table 11.1-21. (cont.).



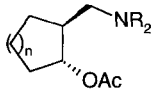


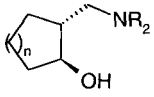
n				
1	98 % ee, 47 % yield, PCL, vinyl acetate	**45a**	>99 % ee, 57 % yield	**45b** [29]
2	>99 % ee, 31 % yield, PCL, vinyl acetate	**46a**	49 % ee, 63 % yield	**46b**[29]



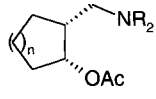


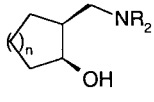
n				
1	99 % ee, 50 % yield, PCL, vinyl acetate	**47a**	99 % ee, 50 % yield	**47b** [30]
2	99 % ee, 47 % yield, CAL-B, isopropenyl acetate	**48a**	70 % ee, 47 % yield	**48b** [30]





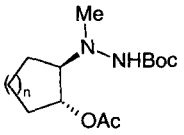
n	R				
1	Me	93–98 % ee, –, CAL-B or PCL, vinyl acetate	**49a**	97–98 % ee, –	**49b** [31]
2	Me	98 % ee, 42 % yield, CAL-B, vinyl acetate	**50a**	96 % ee, 34 % yield	**50b** [32]
3	Me	95 % ee, –, CAL-B or PCL, vinyl acetate	**51a**	92–95 % ee, –	**51b** [31]
1	-$(CH_2)_5$-	97–99 % ee, –, CAL-B or PCL, vinyl acetate	**52a**	99 % ee, –	**52b** [31]
2	-$(CH_2)_5$-	99 % ee, 46 % yield, CAL-B, vinyl acetate	**53a**	97 % ee, 49 % yield	**53b** [32]
2	CH_2Ph	>99 % ee, 40 % yield, PCL, vinyl acetate	**54a**	99 % ee, 50 % yield	**54b** [32]

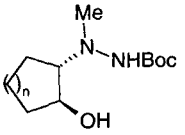




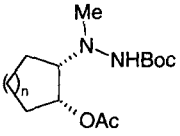
n	R				
1	Me	95–99 % ee, –, PCL or CAL-B, vinyl acetate	**55a**	94–99 % ee, –	**55b** [31]
2	Me	99 % ee, 38 % yield, PCL, vinyl acetate	**56a**	96 % ee, 49 % yield	**56b** [32]
2	Me	94–96 % ee, –, PCL or CAL-B, vinyl acetate	**57a**	37–53 % ee, –	**57b** [31]
2	–$(CH_2)_5$-	98 % ee, 44 % yield, PCL, vinyl acetate	**58a**	97 % ee, 46 % yield	**58b** [32]

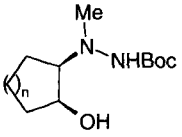
Table 11.1-21. (cont.).



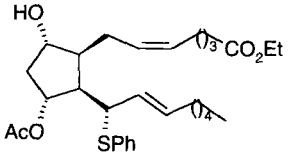


n				
1	91 % ee, 45 % yield, CAL-B, vinyl acetate	**59a**	99 % ee, 31 % yield	**59b** [33]
2	99 % ee, 20 % yield, CAL-B, vinyl acetate	**60a**	99 % ee, 22 % yield	**60b** [33]

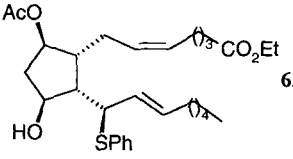




n				
1	99 % ee, 46 % yield, PCL, vinyl butyrate	**61a**	99 % ee, 27 % yield	**61b** [33]
2	>99 % ee, 43 % yield, PCL, vinyl acetate	**62a**	98 % ee, 45 % yield	**62b** [33]



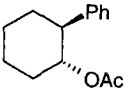
63a [34]



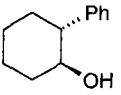
63b [34]

>99 % ee, 42 % yield, PSL, vinyl acetate

>99 % ee, 48 % yield



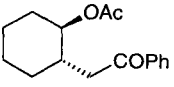
64a [35]



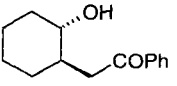
64b [35]

>99 % ee, 50 % yield, PCL, vinyl acetate

>99 % ee, 49 % yield



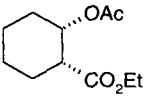
65a [36]



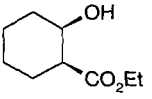
65b [36]

>99 % ee, 44 % yield, PCL, vinyl acetate

>99 % ee, 48 % yield



66a [37]

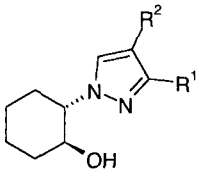


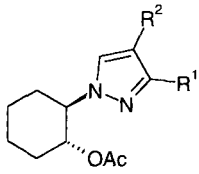
66b [37]

>99 %, 45 % yield, PFL, vinyl acetate, 45 % conversion

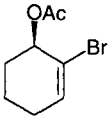
>99 % ee, –

Table 11.1-21. (cont.).



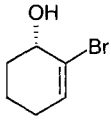


R^1	R^2				
Me	H	91 % ee, –, CAL-B, vinyl acetate	**67a**	–, –	**67b** [38]
t-Bu	H	93 % ee, –, CAL-B, vinyl acetate	**68a**	–, –	**68b** [38]
c-C_6H_{11}	H	85 % ee, –, CAL-B, vinyl acetate	**69a**	–, –	**69b** [38]
Ph	H	89 % ee, –, CAL-B, vinyl acetate	**70a**	–, –	**70b** [38]
-$(CH_2)_3$-		95 % ee, –, CAL-B, vinyl acetate	**71a**	–, –	**71b** [38]
-$(CH_2)_4$-		96 % ee, –, CAL-B, vinyl acetate	**72a**	–, –	**72b** [38]
-$(CH_2)_5$-		98 % ee, –, CAL-B, vinyl acetate	**73a**	–, –	**73b** [38]



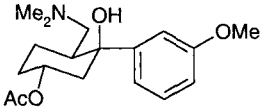
95 % ee, –, PPL, vinyl acetate, 44 % conversion

74a [39]



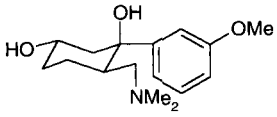
75 % ee, –

74b [39]



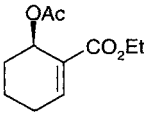
99 % ee, –, CRL, isopropenyl acetate, 34 % conversion

75a [40]



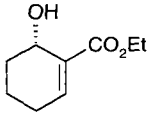
60 % ee, –

75b [40]



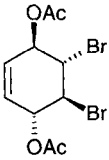
99 % ee, 40 % yield, PCL, vinyl acetate

76a [41]



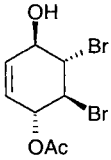
95 % ee, 53 % yield

76b [41]



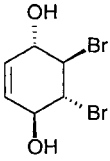
>98 % ee, 10 % yield, MML, vinyl acetate

77a [42]



>98 % ee, 37 % yield

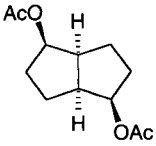
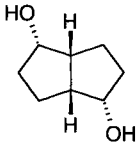
77b [42]

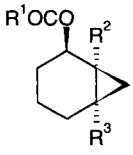


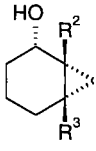
>98 % ee, 48 % yield

77c [42]

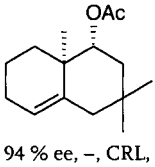
Table 11.1-21. (cont.).

			
96 % ee, 38 % yield, PCL, vinyl acetate	**78a** [43]	98 % ee, 44 % yield	**78b** [43]
>99 % ee, 32 % yield, pancreatin, $AcOCH_2CCl_3$	**78a** [43]	55 % ee, 57 % yield	**78b** [44]

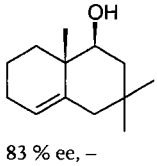




R^1	R^2	R^3				
Me	H	H	84 % ee, –, CAL-B, 54 % conversion	**79a**	98 % ee, –	**79b** [45]
Me	H	Me	86 % ee, –, CAL-B, 53 % conversion	**80a**	99 % ee, –	**80b** [45]
Me	Me	H	90 % ee, –, CAL-B, 52 % conversion	**81a**	97 % ee, –	**81b** [45]
n-C_5H_{11}	H	H, alkyl, CH_2Ph	87– >99 % ee, 43–51 % yield, CAL-B, isopropenyl hexanoate	**82a**	75– >99 % ee, 41–53 % yield	**82b** [46]



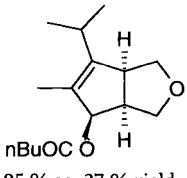
83a [47]



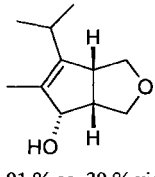
83b [47]

94 % ee, –, CRL, vinyl acetate

83 % ee, –



84a [48]



84b [48]

95 % ee, 27 % yield, PSL, vinyl butanoate, double resolution

91 % ee, 29 % yield

Table 11.1-21. (cont.).

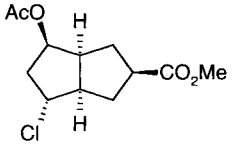
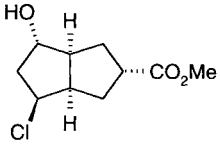
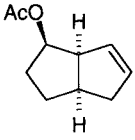
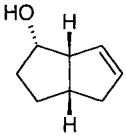
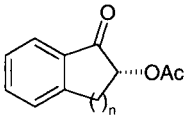
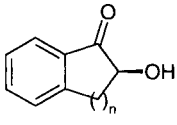
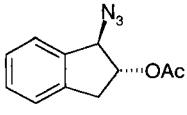
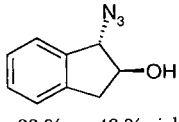
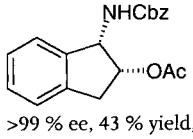
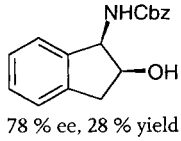
	**85a** [49]		**85b** [49]
>97 % ee, 43 % yield, PCL, vinyl acetate		>97 % ee, 42 % yield	
	**86a** [50]		**86b** [50]
99 % ee, 44 % yield, PSL, vinyl acetate		98 % ee, 38 % yield	
99 % ee, 41 % yield, PCL, vinyl acetate		92 % ee, 32 % yield	
			
n			
1 86 % ee, –, PCL, vinyl acetate, 52 % conversion	**87a** [51]	95 % ee, –	**87b**[51]
2 79 % ee, –, PSL, vinyl acetate, 57 % conversion	**88a** [51]	99 % ee, –	**88b** [51]
			
98 % ee, 48 % yield, PCL, vinyl acetate	**89a** [52]	>99 % ee, 48 % yield	**89b** [52]
>96 % ee, 44% yield, PCL, isopropenyl acetate	**89a** [53]	>96 % ee, 46% yield	**89b** [53]
	**90a** [54]		**90b** [54]
>99 % ee, 43 % yield, PSL, vinyl acetate		78 % ee, 28 % yield	

Table 11.1-21. (cont.).

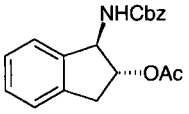
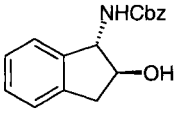
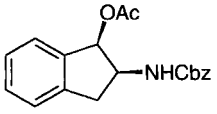
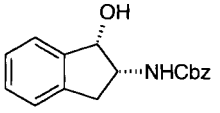
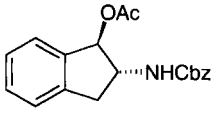
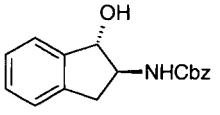
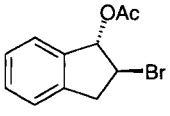
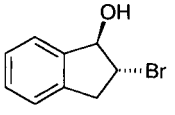
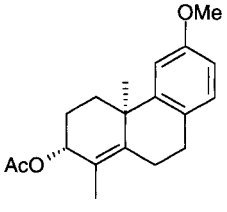
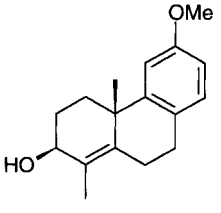
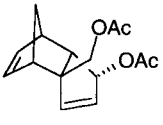
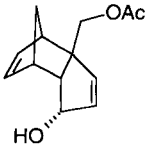
	**91a** [54]		**91b** [54]
98 % ee, 41 % yield, PSL, vinyl acetate		>99 % ee, 37 % yield	
	**92a** [54]		**92b** [54]
>99 % ee, 41 % yield, PSL, vinyl acetate		96 % ee, 38 % yield	
	**93a** [54]		**93b** [54]
95 % ee, 38 % yield, PSL, isopropenyl acetate		20 % ee, 40 % yield	
	**94a** [55]		**94b** [55]
93 % ee, 35 % yield, CAL-B, vinyl acetate		100 % ee, 31 % yield	
	**95a** [56]		**95b** [56]
88 % ee, 48 % yield, CAL-B, vinyl acetate		98 % ee, 40 % yield	
	**96a** [57]		**96b** [57]
92 % ee, 41 % yield, LIP, vinyl acetate		>99 % ee, 46 % yield	

Table 11.1-21. (cont.).

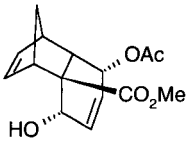
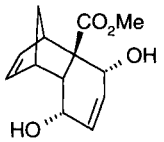
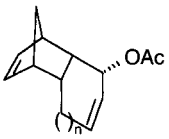
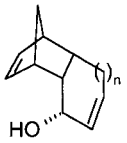
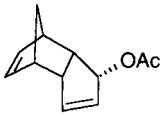
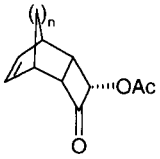
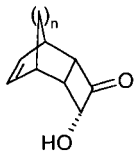
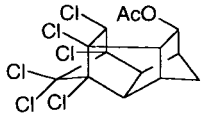
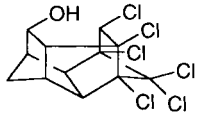
	**97a** [58]		**97b** [58]
>99 % ee, 44 % yield, CAL-B, vinyl acetate		98 % ee, 47 % yield	
			
n			
1	99 % ee, 42 % yield, LIP, vinyl acetate	**98a** [59]	95 % ee, 43 % yield	**98b** [59]
2	94 % ee, 36 % yield, LIP, vinyl acetate	**99a** [59]	55 % ee, 43 % yield	**99b** [59]
	**100a** [60]		**100b** [60]
>99 % ee, 49 % yield, PCL, vinyl acetate		>99 % ee, 50 % yield	
			
n			
1	>99 % ee, 50 % yield, PCL, vinyl acetate	**101a** [61]	>99 % ee, 49 % yield	**101b** [61]
2	>99 % ee, 46 % yield, PCL, vinyl acetate	**102a** [61]	>99 % ee, 49 % yield	**102b** [61]
	**103a** [62]		**103b** [62]
>95 % ee, –, CRL, vinyl acetate, 44 % conversion		77 % ee, –	

Table 11.1-21. (cont.).

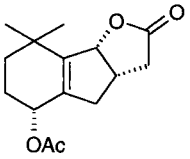
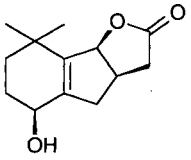
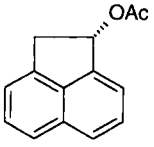
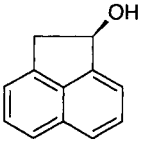
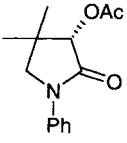
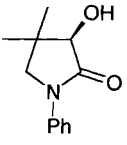
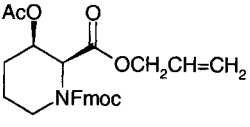
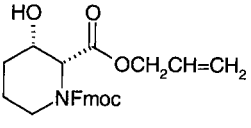
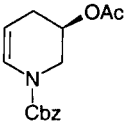
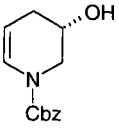
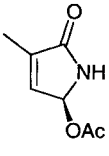
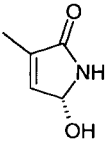
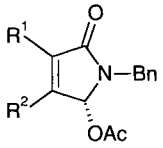
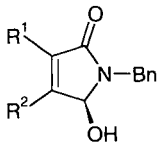
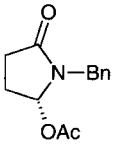
 87 % ee, 52 % yield, PSL, vinyl acetate	**104a** [63]	 >98 % ee, 48 % yield	**104b** [63]
 94 % ee, 41 % yield, PFL, isopropenyl acetate	**105a** [64]	 97 % ee, 34 % yield	**105b** [64]
 88 % ee, 46 % yield, PCL, vinyl acetate	**106a** [65]	 99 % ee, 46 % yield	**106b** [65]
 96 % ee, 46 % yield, PCL, vinyl acetate	**107a** [66]	 99 % ee, 43 % yield	**107b** [66]
 >99 % ee, 47 % yield, PCL, vinyl acetate	**108a** [67]	 >99 % ee, 48 % yield	**108b** [67]
 50 % ee, 53 % yield, lipase PL, vinyl acetate	**109a** [68]	 98 % ee, 35 % yield	**109b** [68]

Table 11.1-21. (cont.).



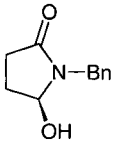


R^1	R^2			
H	H	>99 % ee, 50 % yield, PCL or CAL-B, vinyl acetate	>99 % ee, 48 % yield	**110b** [69]
Me	H	>99 % ee, 49 % yield, CAL-B, vinyl acetate	>99 % ee, 48 % yield	**111b** [69]



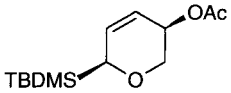
112a [69]

88 % ee, 49 % yield, PCL, vinyl acetate



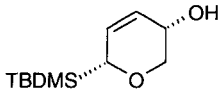
112b [69]

99 % ee, 37 % yield



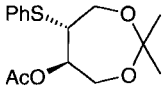
113a [70]

78 % ee, 52 % yield, PCL, vinyl acetate
78 % ee, 54 % yield, PSL, vinyl acetate



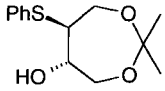
113b [70]

97 % ee, 40 % yield
99 % ee, 42 % yield



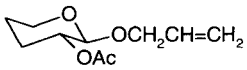
114a [71]

>99 % ee, 49 % yield, PCL, vinyl acetate



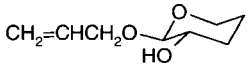
114b [71]

>99 % ee, 49 % yield



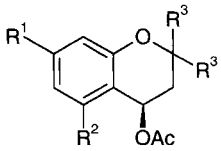
115a [72]

62 % ee, 52 % yield, PCL, vinyl acetate



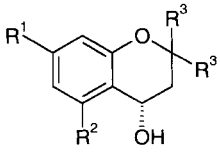
115b [72]

93 % ee, 96 % yield



116a [73]

R^1 = H, Me, R^2 = H, OMe, R^3 = H, Me
71–100 % ee, –, CCL, vinyl acetate, 19–57 % conversion



116b [73]

22–100 % ee, –

Table 11.1-21. (cont.).

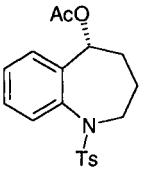
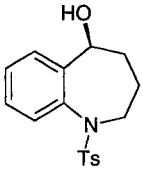
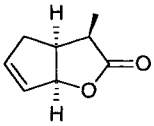
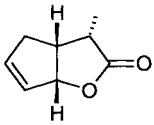
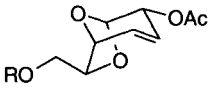
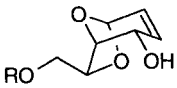
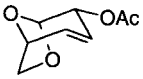
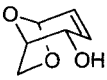
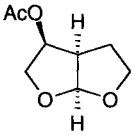
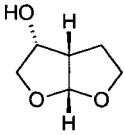
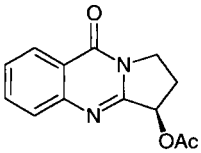
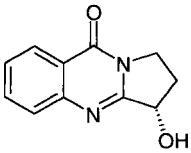
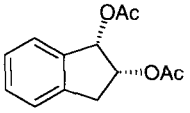
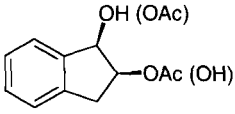
	**117a** [74]		**117b** [74]
96 % ee, 25 % yield, ASL, vinyl acetate		32 % ee, 75 % yield	
	**118a** [75]		**118b** [75]
92 % ee, 50 % yield, PFL, vinyl acetate		100 % ee, 47 % yield	
	**119a** [76]		**119b** [76]
R = 2-Naphthylmethyl, CH_2Ph, TBDMS >99 % ee, 47–50 % yield, PCL, vinyl acetate		>99 % ee, 47–49 % yield	
	**120a** [77]		**120b** [77]
>99 % ee, 48 % yield, PSL, vinyl acetate		>99 % ee, 47 % yield	
	**121a** [78]		**121b** [78]
87 % ee, 45 % yield, PCL, acetic anhydride		95 % ee, 42 % yield	
	**122a** [79]		**122b** [79]
>99 % ee, –, PCL, vinyl acetate, 45 % conversion		–, –	

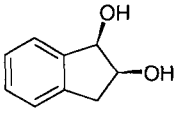
Table 11.1-21. (cont.).



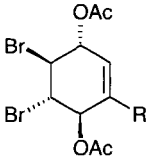
123a [86]
100 % ee, 38 % yield
PCL, vinyl acetate

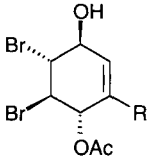


123b [86]
100 % ee, 36 % yield

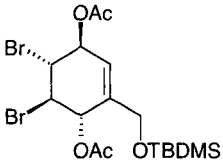


123c [80]
100 % ee, 10 % yield



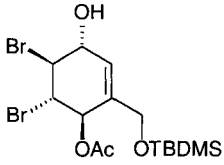


R				
H	>98 % ee, 50 % yield	**124a**	92 % ee, 50 % yield	**124b** [81]
Br	94 % ee, 49 % yield	**125a**	87 % ee, 50 % yield	**125b** [81]
Me	87 % ee, 50 % yield	**126a**	85 % ee, 50 % yield	**126b** [81]
	all PCL, vinyl acetate			



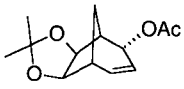
127a [81]

>98 % ee, 47 % yield, CAL-B
vinyl acetate



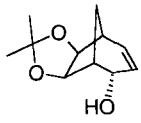
127b [81]

92 % ee, 50 % yield



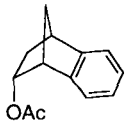
128a [82]

96 % ee, 49 % yiel, PCL,
vinyl acetate



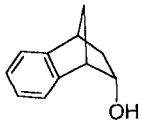
128b [82]

95 % ee, 49 % yield



129a [83]

>99 % ee, 45 % yield, CAL-B,
vinyl acetate, 50 °C



129b [83]

>99 % ee, 46 % yield

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28, **29**, **31–33**, **41–65**, **67–71**, **83–89**, **107–109**, **127**, **143**, **144**, **153**, **154**, **156**, **157**, **163–170**, **181–183**, **201**) (Table 11.1-20). A very good illustration for the potential of enantiomer-differentiating acylation catalyzed by lipases is provided by the high-yield synthesis of a series of aromatic cyanohydrin acetates (**1a–g**) from aldehydes, acetone cyanohydrin and vinyl acetate in the presence of *Pseudomonas cepacia* lipase and a basic anion-exchange resin in diisopropyl ether which proceeds under kinetic resolution coupled with *in situ* formation and racemization of the cyanohydrin representing a dynamic kinetic resolution. For further examples see Table 11.1-24.

To the secondary aliphatic alcohols, which have been resolved into their enantiomers, belong a variety of hydroxy carboxylic esters and acids (**35**, **100–102**, **125**, **126**, **131–140**, **150**, **151**, **166**, **168–172**, **174**, **182**, **183**), some hydroxy ketones (**128–130**, **141**) and a crown ether derivative (**203**) (Table 11.1-20). Even the tetraphenylporphyrin derivatives **204** and **205** were substrates for different lipases.

Diketene is useful acyl donor also, yielding acetoacetates with very high enantiomeric excess (**115**, **207–210**).

Monoacetates and alcohols of Table 11.1-20 which can be obtained with other hydrolases as such or of opposite configuration are contained in Tables 11.1-6 and 11.1-15.

Table 11.1-21 lists cyclic secondary alcohols that have been synthesized by lipase-catalyzed enantiomer-differentiating acylation (**1–129**). The compounds that have been obtained by the alternative route of hydrolysis are listed in Table 11.1-16. The complementary nature of the two routes is obvious. For the series of the glycals **9–15**, *Pseudomonas cepacia* lipase-catalyzed acylation works with good to high enantiomer selectivity and yield. *myo*-Inositol derivatives **17** and **18** may be prepared enantiomer-

ically pure by *Candida cylindracea* lipase-catalyzed acylation with acetic anhydride in diethyl ether not only with high enantiomer but also with high group selectivity.

Axial-chiral enantiomerically highly enriched binaphthols **4**, which are highly useful chiral auxiliaries, are accessible either through acylation of the racemic diol with vinyl acetate or deacylation of the racemic diacetate with butanol (Table 11.1-22), both catalyzed by *Pseudomonas cepacia* lipase.

Among the many other cyclic secondary alcohols that have been obtained by lipase-catalyzed enantiomer-selective acylation with high enantiomeric excess are aminofunctionalized cycloalkanols (**40**, **45–62**, **75**), bicyclo[3.3.0]octanols (**78**, **84–86**), different types of tri- and tetracyclic alcohols (**96–104**), substituted indanols (**87–94**, **123**), hydroxy lactams (**106**, **109–112**) and brominated cyclohexenol derivatives (**74**, **77**, **124–127**) (Table 11.1-21).

Monoacetates and alcohols of Table 11.1-21 which can be obtained with other hydrolases as such or of opposite configuration are contained in Tables 11.1-6 and 11.1-16.

11.1.1.3
Inter- and Intramolecular Alcoholysis

Hydrolase-catalyzed enantiomer-differentiating alcoholysis of esters of racemic alcohols with achiral alcohols in organic solvents of low water content is a valuable alternative to hydrolysis (Table 11.1-22).

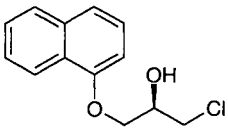
Lipase-catalyzed enantiomer-differentiating inter- and intramolecular alcoholysis of acylated alcohols and lactones in organic solvents may most advantageously be used instead of hydrolysis in aqueous solution in those cases where insufficient stability, high solubility or low functional group selectivity is observed or may be anticipated in the latter case (**1–16**) (Table 11.1-22).

For lipase-catalyzed intermolecular alcoholysis as alcohols, by and large more lipophilic ones such as *n*-propanol, *n*-butanol, *n*-hexanol, *n*-octanol, cyclohexanol or benzylalcohol are used whereas methanol (**44**) or ethanol (**52**, **53**, **61**) are used rarely. Typical solvents are *n*-hexane, diisopropyl ether, *tert*-pentyl alcohol, toluene, tetrahydrofuran or acetonitrile. In many cases the enantioselectivity and yield are higher for the alcoholysis than for the hydrolysis catalyzed by one and the same lipase, provided that a large excess of the alcohol is used. Enantiomer-differentiating alcoholysis of an acylated thiol (**10**) has also been described.

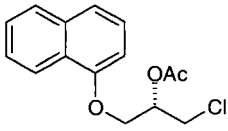
Alcoholysis of γ-and β-lactones gives access to enantiomerically pure γ-hydroxyesters and γ-lactones (**17–24**) and β-hydroxyesters and β-lactones (**55–63**), respectively. Enantiomerically pure enol acetates **67a** and the γ-acetoxybutenolide **51**/*ent*-**51** have been obtained by hydrolysis of the corresponding racemic substrates.

As well as the above-described intermolecular alcoholysis of esters, the intramolecular version has been successfully utilized for the synthesis of lactones from racemic hydroxy carboxylic acid esters (**25–41**, **64–66**) (Table 11.1-22). High selectivity in the pig pancreas lipase-catalyzed enantiomer-differentiating lactonization of γ-hydroxy carboxylic acid esters with formation of butyrolactones substituted in

Table 11.1-22. Lipase-catalyzed enantiomer- and enantiotopos-differentiating inter- and intramolecular alcoholysis of esters and lactones in organic solvents (PCL *Pseudomonas cepacia* lipase, PSL *Pseudomonas* sp. lipase, PPL pig pancreas lipase, MML *Mucor miehei* lipase, HLL, *Humicola lanuginosa* lipase, PFL *Pseudomonas fluorescens* lipase, CCL *Candida cylindracea* lipase, CAL-B *Candida antarctica* B lipase, CRL *Candida rugosa* lipase, PRL *Penicillium roqueforti* lipase, CAL-A+B *Candida antarctica* A+B lipase).

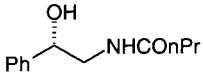


1a [1]

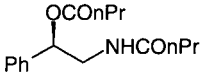


1b [1]

≥95 % ee, 45 % yield	BuOH	90 % ee, 45 % yield
≥95 % ee, 45 % yield	BuOH/*i*-Pr_2O	≥95 % ee, 45 % yield
≥95 % ee, 45 % yield	H_2O	88 % ee, 45 % yield
all PCL		

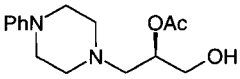


2a [2]

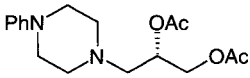


2b [2]

≥95 % ee, 34 % yield	BuOH, *t*-pentanol	≥95 % ee, 33 % yield
≥95 % ee, 39 % yield	BuOH, toluene	≥95 % ee, 40 % yield
≥95 % ee, 42 % yield	HexOH, toluene	≥95 % ee, 46 % yield
≥95 % ee, 43 % yield	OctOH, toluene	≥95 % ee, 41 % yield
≥95 % ee, 45 % yield	BuOH, *n*-Bu_2O	≥95 % ee, 39 % yield
≥95 % ee, 36 % yield	HexOH, *n*-Bu_2O	≥95 % ee, 41 % yield
≥95 % ee, 44 % yield	BuOH, THF	79 % ee, 45 % yield
≥95 % ee, 43 % yield	BuOH, MeCN	85 % ee, 46 % yield
all PCL		

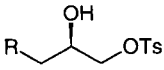


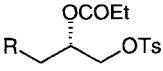
3a [3]



3b [3]

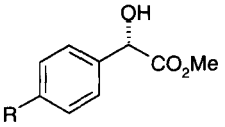
95 % ee, 47 % yield, PCL	*n*-PrOH	95 % ee, 50 % yield
CCl_4, *t*-pentanol, *i*-Pr_2O		





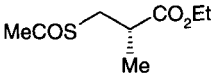
R					
$ClCH_2$	96 % ee,–, PFL, BuOH, *n*-hexane	**4a**	96 % ee,–,	**4b** [4]
n-Bu	90 % ee,–, PFL, BuOH, *n*-hexane	**5a**	31 % ee,–	**5b** [4]
n-$C_{10}H_{21}$	≥98 % ee,–, PFL, BuOH, *n*-hexane	**6a**	43 % ee,–	**6b** [4]
Ph	95 % ee,–, CCL, BuOH, *n*-hexane	**7a**	70 % ee,–	**7b** [4]
$PhOCH_2$	84 % ee,–, HLL, BuOH, *n*-hexane	**8a**	42 % ee,–	**8b** [4]

Table 11.1-22. (cont.).



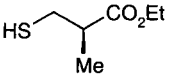
9 [5, 6]

R = H, F, Cl, Br, OMe
97–99 % ee, CCL, BuOH, *i*-Pr_2O
(from the butyrate)



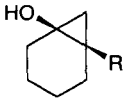
10a [7]

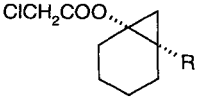
88 % ee, 39 % yield, *n*-PrOH, PPL, hexane



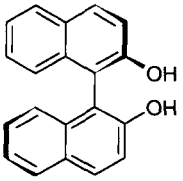
10b [7]

95 % ee, 42 % yield



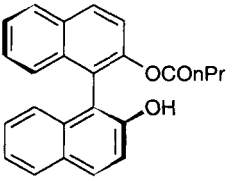


R				
H	79 % ee, 44 % yield	**11a**	84 % ee, 45 % yield	**11b** [8]
Me	72 % ee,–	**12a**	95 % ee,–	**12b** [8]
Et	80 % ee,–	**13a**	91 % ee,–	**13b** [8]
n-Bu	86 % ee,–	**14a**	90 % ee,–	**14b** [8]
n-C_6H_{13}	66 % ee,–	**15a**	86 % ee,–	**15b** [8]
	all MML, PrOH, *i*-Pr_2O			



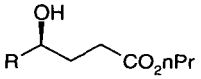
16a [9]

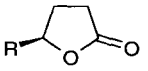
98 % ee, 47 % yield, PSL, BuOH



16b [9]

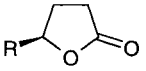
96 % ee, 50 % yield



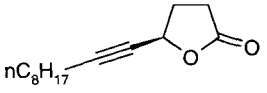


R				
n-C_5H_{11}	70 % ee, –	**17a**	55 % ee, –	**17b** [10]
n-C_6H_{13}	90 % ee, –	**18a**	78 % ee, –	**18b** [10]
n-C_7H_{15}	93 % ee, –	**19a**	80 % ee, –	**19b** [10]
n-C_8H_{17}	≥98 % ee, –	**20a**	77 % ee, –	**20b** [10]
n-C_9H_{19}	≥98 % ee, –	**21a**	71 % ee, –	**21b** [10]
n-$C_{10}H_{21}$	≥98 % ee, –	**22a**	74 % ee, –	**22b** [10]
n-$C_{11}H_{23}$	≥98 % ee, –	**23a**	76 % ee, –	**23b** [10]
n-$C_{12}H_{25}$	≥98 % ee, –	**24a**	77 % ee, –	**24b** [10]
	all PPL, *n*-PrOH			

Table 11.1-22. (cont.).

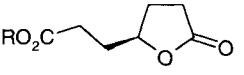


R		
Me	≥98 % ee, –	**25** [11]
Et	88 % ee, –	**26** [11]
n-C_6H_{13}	82 % ee, –	**27** [11]
n-C_8H_{17}	91 % ee, –	**28** [11]
Ph	92 % ee, –	**29** [11]
4-MeC_6H_4	94 % ee, –	**30** [11]
4-$MeOC_6H_4$	88 % ee, –	**31** [11]
4-BrC_6H_4	94 % ee, –	**32** [11]
	all PPL, Et_2O or hexane or THF (intramolecular lactonization of the methyl ester)	



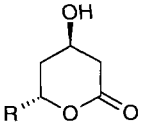
33 [12]

78 % ee, –, PPL, Et_2O (from methyl ester)

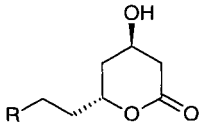


34 [11]

R = Me, Et, $PhCH_2$
≥95 % ee, –, PPL, Et_2O

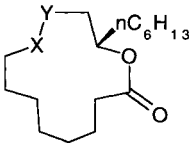


R		
n-C_5H_{11}	86 %ee, 25 % yield	**35** [13]
n-$C_{15}H_{31}$	≥98 % ee, 15 % yield, PPL, Et_2O (from the pentyl ester)	**36** [13]

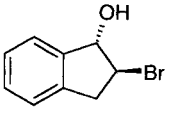


R		
Ph	≥98 % ee, 35 % yield	**37** [14]
c-C_6H_{13}	≥98 % ee, 35 % yield	**38** [14]
	all PPL, Et_2O	

Table 11.1-22. (cont.).

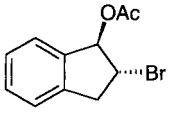


X-Y		
CH_2CH_2	≥99 % ee, 14 % yield	**39** [15]
(*E*)-CH=CH	≥99 % ee, 18 % yield	**40** [15]
(*Z*)-CH=CH	98 % ee, 20 % yield PSL, isooctane, molecular sieves	**41** [15]



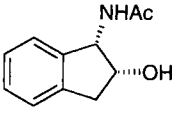
42a [16]

94 % ee, 47 % yield, CAL-B, cyclohexanol



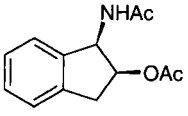
42b [16]

92 % ee, 49 % yield



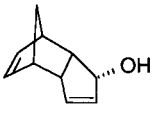
43a [17]

>99 % ee, 43 % yield, CAL-B, *n*-BuOH



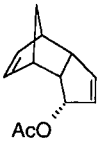
43b [17]

>99 % ee, 48 % yield



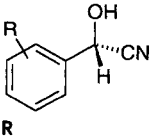
44a [18]

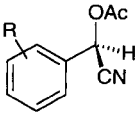
>99 % ee, 44 % yield, PCL, MeOH



44b [18]

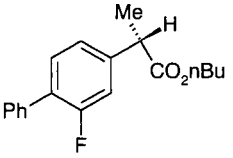
80 % ee, 55 % yield





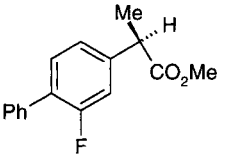
R				
3-OPh	>99 % ee, –, PCL, *n*-BuOH, 49 % conversion	**45a**	97 % ee, –	**45b** [19]
3-OPh	98 % ee, –, CAL-B, *n*-PrOH, 45 % conversion	**45a**	79 % ee, –	**45b** [20]
R = H, 3-Me, 4-Me, 3-OMe, 4-OMe, 3-Cl, 4-Cl	82–98 % ee, –, CAL-B, *n*-PrOH, 45–53 % conversion	**46a**	79- >9 % ee, –	**46b** [20]

Table 11.1-22. (cont.).



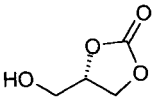
47a [21]

82 % ee, –, CAL-B, *n*-BuOH, 34 % conversion



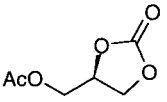
47b [21]

–, –



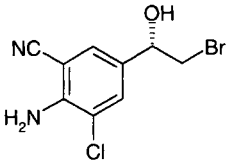
48a [22]

96 % ee, 60 % yield, PFL, *n*-PrOH



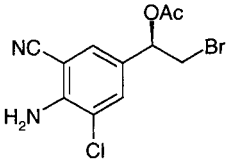
48b [22]

13 % ee, 37 % yield



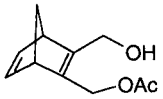
49a [23]

86 % ee, 50 % yield, CAL-B, *n*-BuOH



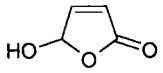
49b [23]

99 % ee, 42 % yield

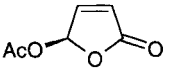


50 [24]

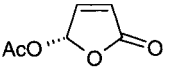
>95 % ee, 93 % yield, PSL, *n*-BuOH



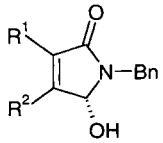
51a [25]
racemate

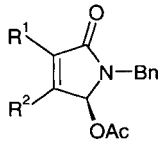


51b [25]



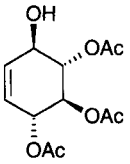
***ent*-51b** [25]
70–98 % ee, –, CCL, lipase PSL, PRL, CRL, PCL, *n*-BuOH, 49–61 % conversion



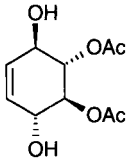


R^1	R^2				
H	H	>99 % ee, 45 % yield, PCL, EtOH	**52a**	90 % ee, 49 % yield 43 % ee, 62 % yield	**52b** [26]
Me	H	>99 % ee, 26 % yield, PCL, EtOH	**53a**		**53b** [26]

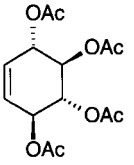
Table 11.1-22. (cont.).



54a [27]



54b [27]

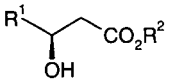


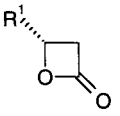
54c [27]

>98 % ee, 24 % yield, CCL, *n*-BuOH

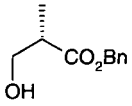
–, 18 % yield

68 % ee, 58 % yield

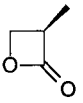




R^1	R^2				
Me	CH_2Ph	85 % ee, 51 % yield, PPL, $PhCH_2OH$	**55a**	96 % ee, 36 % yield	**55b** [28]
n-Pr	CH_2Ph	69 % ee, 45 % yield, PCL, $PhCH_2OH$	**56a**	75 % ee, 42 % yield	**56b** [28]
i-Pr	CH_2Ph	90 % ee, 43 % yield, PCL, $PhCH_2OH$	**56a**	95 % ee, 41 % yield	**56b** [28]



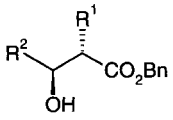
57a [28b]

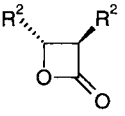


57b [28b]

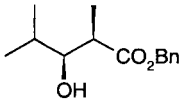
72 % ee, 24 % yield, PCL, $PhCH_2OH$

70 % ee, 38 % yield

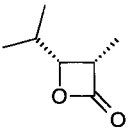




R^1	R^2				
Me	*n*-Pr	87 % ee, 34 % yield, PCL, $PhCH_2OH$	**58a**	92 % ee, 50 % yield	**58b** [28b]
n-Pr	Me	98 % ee, 24 % yield, PCL, $PhCH_2OH$	**59a**	79 % ee, 16 % yield	**59b** [28b]



60a [28b]



60b [28b]

84 % ee, 39 % yield, PCL, $PhCH_2OH$

85 % ee, 13 % yield

Table 11.1-22. (cont.).

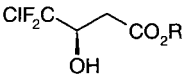
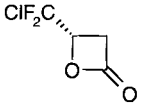
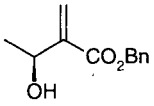
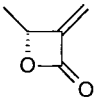
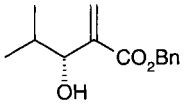
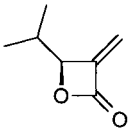
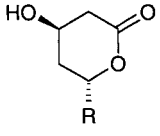
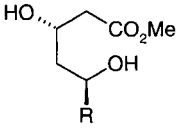
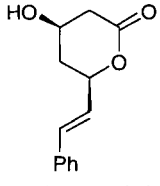
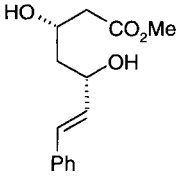
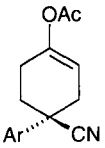
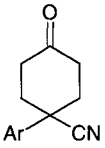
				
R				
Et	81 % ee, 40 % yield, PCL, EtOH	**61a**	97 % ee, 43 % yield	**61b** [29]
n-C_6H_{13}	73 % ee, 36 % yield, PCL, n-$C_6H_{13}OH$	**61a**	>99.9 % ee, 39 % yield	**61b** [29]
		**62a** [30]		**62b** [30]
95 % ee, –, CAL-B, $PhCH_2OH$, 51 % conversion			99 % ee, –	
		**63a** [30]		**63b** [30]
99 % ee, –, CAL-B, $PhCH_2OH$, 50 % conversion			>99 % ee, –	
				
R				
E-CH=CH-Ph	80 % ee, 33 % yield, pancreatin, >99 % ee, double resolution	**64a**	–, 47 % yield	**64b** [31]
C≡CH-Ph	61 % ee, –, CAL-A+B, 98 % ee, by crystallization from the reaction mixture	**65a**	70 % ee, 30 % yield	**65b** [32]
		**66a** [33]		**66b** [33]
96 % ee, 28 % yield, CAL-A+B			–, –	

Table 11.1-22. (cont.).

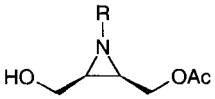


67a [34]

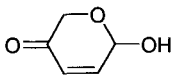
100 % ee, 38 % yield (Ar = Ph), 30 % yield (Ar = 3,4-Cl_2Ph), PFL, *n*-BuOH



67b [34]

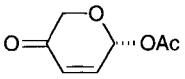


R		
Ts	95 % ee, 76 % yield, PCL, *n*-BuOH	**68** [35]
	72 % ee, 56 % yield, CCL, *n*-BuOH	
Cbz	98 % ee, 68 % yield, PCL, *n*-BuOH	**69** [35]
	49 % ee, 30 % yield, PPL, *n*-BuOH	



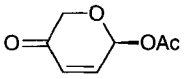
racemic

70a [36]



>99 % ee, 65 % conversion, PCL, *n*-BuOH, *n*-hexane

70b [36]



>99 % ee, 57 % conversion, CCL, *n*-BuOH, *n*-hexane

***ent*-70b** [36]

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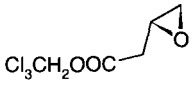
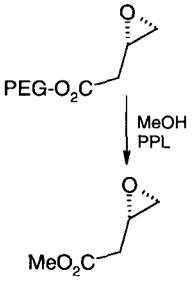
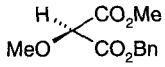
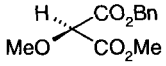
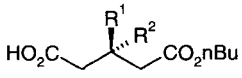
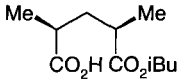
4-position and the unchanged γ-hydroxy carboxylic acid esters of opposite configuration were observed (**25–34**). Pig pancreas lipase in diethyl ether is the combination of choice. Formation of the corresponding monosubstituted γ-valerolactones was unselective. γ-Valerolactones with a hydroxyl group in 4-position however could be obtained with high selectivity from the corresponding dihydroxy carboxylic acid pentyl or methyl ester (**35–38**). In order to suppress the competition between the methanol formed during lactonization and the intramolecular hydroxyl group, reactions were run in the presence of molecular sieve. Otherwise, conversion and *ee* value of the lactone were poor because of the reversibility of the reaction. Interestingly, macrocyclic lactones may be prepared by this method too. Treatment of racemic ricinoleic acid methyl ester, its racemic *trans*-isomer and the saturated racemic derivative with *Pseudomonas* sp. lipase in isooctane in the presence of molecular sieve gave the corresponding (*R*)-configurated 13-membered lactones **39–41** (Table 11.1-22) in fair yields with high *ee* values.

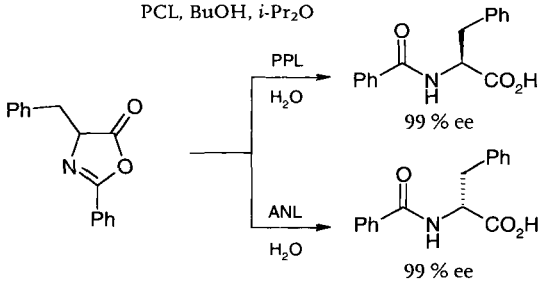
Acylated alcohols, alcohols and lactones of Table 11.1-22 which can be obtained with other hydrolases as such or of opposite configuration are contained in Tables 11.1-14 to 11.1-16 and Tables 11.1-19 to 11.1-21.

Lipase-catalyzed enantiomer- and enantiotopos-differentiating alcoholysis may also be extended to carboxylic acid esters, anhydrides and oxazolin-2-ones (**1–22**) (Table 11.1-23). Alcoholysis of methoxy malonic acid dimethyl ester with benzyl alcohol catalyzed by *Candida cylindracea* lipase gave, at 50% conversion, the mixed diester **2** with high enantioselectivity. At higher conversion the *ee* values are lower because of the reversibility of alcoholysis. The enantiomeric mixed diester *ent*-**2** may be obtained by methanolysis of the corresponding dibenzyl ester. Through catalytic hydrogenolysis the monobenzyl ester can be converted into the corresponding acid. It remains to be shown if this is an alternative to the pig liver esterase or lipase-catalyzed hydrolysis of the corresponding prochiral diester (Table 11.1-2).

Table 11.1-23. Lipase-catalyzed enantiomer- and enantiotopos-differentiating alcoholysis of carboxylic acid esters and anhydrides, alcoholysis or hydrolysis of oxazolin-2-ones, and esterification of carboxylic acids (PPL pig pancreas lipase, PCL *Pseudomonas cepacia* lipase, ANL *Aspergillus niger* lipase, CSL *Candida* sp. lipase, *Candida cylindracea* lipase, CAL-B *Candida antarctica* B lipase, CRL *Candida rugosa* lipase).

	**1a** [1]		**1b** [1]
≥96 % ee, 43 % yield, PPL PEG, *i*-Pr_2O		≥89 % ee, 46 % yield	
	**2** [2]		***ent*-2** [2]
absolute configuration unknown ≥96 % ee, –, CCL $PhCH_2OH$, hexane (from the dimethyl ester)		absolute configuration unknown 90 % ee, –, CCL equlibrium (70 %) MeOH, hexane, 50 % conversion (from the dibenzyl ester)	
			**8** [5]

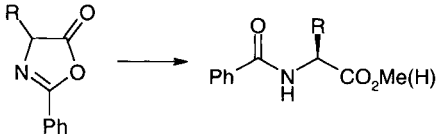
R^1	R^2			
Me	H	93 % ee	**3** [3, 4]	90 % ee, 72 % yield
Et	H	87 % ee	**4** [3, 4]	CSL, *i*-BuOH,
n-Pr	H	60 % ee	**5** [3, 4]	*c*-C_6H_{12}
i-Pr	H	76 % ee	**6** [3, 4]	
H	Cl	62 % ee	**7** [3, 4]	
		65–95 % yield		
		PCL, BuOH, *i*-Pr_2O		



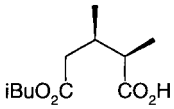
9 [6]

***ent*-9** [6]

Table 11.1-23. (cont.).

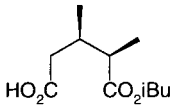


R		
Me_2CH	77 % ee, 47 % yield (H_2O)	**10** [7–9]
Me_2CHCH_2	78 % ee, 82 % yield	**11** [7–9]
$MeS(CH_2)_2$	82 % ee, 31 % yield (H_2O)	**12** [7–9]
2-Naphthylmethyl	75 % ee, 90 % yield	**13** [7–9]
4-$MeC_6H_4CH_2$	66 % ee, 86 % yield	**14** [7–9]
Ph	75 % ee, 46 % yield (H_2O)	**15** [7–9]
$PhCH_2$	69 % ee, 93 % yield	**16** [7–9]
$Ph(CH_2)_2$	93 % ee, 61 % yield (H_2O)	**17** [7–9]
$Ph(CH_2)_3$	84 % ee, 91 % yield	**18** [7–9]
	all PCL, MeOH (or H_2O), *t*-BuOMe	



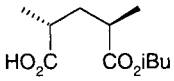
74 % ee, 30 % yield, CAL-B, 2-methylpropanol

19a [10]



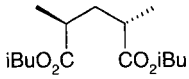
92 % ee, 29 % yield

19b [10]



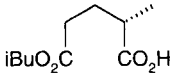
90 % ee, 40 % yield, CAL-B, 2-methylpropanol

20a [11]



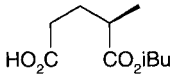
90 % ee, 48 % yield

20b [11]



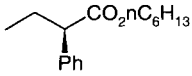
88 % ee, 28 % yield, CAL-B, 2-methylpropanol

21a [12]



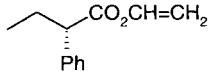
99 % ee, 29 % yield

21b [12]



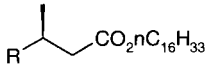
99 % ee, 38 % yield, CAL-B, *n*-hexanol

22a [13]



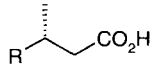
74 % ee, 45 % yield

22b [13]



82–90 % ee, –, CRL, hexadecan-1-ol, 25–39 % conversion, esterification

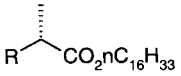
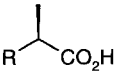
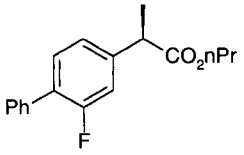
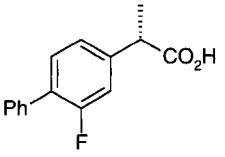
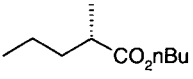
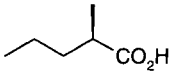
23a [14]



30–50 % ee, –

23b [14]

Table 11.1-23. (cont.).

	**24a** [14]		**24b** [14]
85–93 % ee, –, CRL, hexadecan-1-ol, 15–33 % conversion, esterification		16–45 % ee, –	
	**25a** [15]		**25b** [15]
64 % ee, 58 % yield, CAL-B, *n*-PrOH, HC(O*n*Pr)$_3$		>98 % ee, 39 % yield	
	**26a** [15]		**26b** [15]
53 % ee, 65 % yield, CRL, *n*-BuOH, HC(O*n*Bu)$_3$		>97 % ee, 35 % yield	

1 J. S. Wallace, K. B. Reda, M. E. Williams, C. J. Morrow, *J. Org. Chem.* **1990**, *55*, 3544.
2 A. L. Gutman, M. Shapira, A. Boltanski, *J. Org. Chem.* **1992**, *57*, 1063.
3 Y. Yamamoto, K. Yamamoto, T. Nishioka, J. Oda, *Agric. Biol. Chem.* **1988**, *52*, 3087.
4 Y. Yamamoto, T. Nishioka, J. Oda, *Tetrahedron Lett.* **1988**, *29*, 1717.
5 R. Ozegowski, A. Kunath, H, Schick, *Tetrahedron: Asymmetry* **1993**, *4*, 695.
6 R.-L. Gu, I.-S. Lee, C. J. Sih, *Tetrahedron Lett.* **1992**, *33*, 1953.
7 J. Z. Crich, R. Brieva, P. Marquart, R.-L. Gu, S. Flemming, C. J. Sih, *J. Org. Chem.* **1993**, *58*, 3252.
8 H. S. Bevinakatti, A. A. Banerji, R. V. Newadkar, A. A. Mokashi, *Tetrahedron: Asymmetry* **1992**, 3, 1505.
9 H. S. Bevinakatti, R. V. Newadkar, A. A. Banerji, *J. Chem. Soc., Chem. Commun.* **1990**, 1091.
10 R. Ozegowski, A. Kunath, H. Schick, *Liebigs Ann. Chem.* **1996**, 1443.
11 R. Ozegowski, A. Kunath, H. Schick, *Liebigs Ann. Chem.* **1994**, 215.
12 R. Ozegowski, A. Kunath, H. Schick, *Liebigs Ann. Chem.* **1994**, 1019.
13 H. Yang, E. Henke, U. T. Bornscheuer, *J. Org. Chem.* **1999**, *64*, 1709.
14 B.-V. Nguyen, E. Hedenström, *Tetrahedron: Asymmetry* **1999**, *10*, 1821.
15 R. Morrone, M. Piatelli, G. Nicolosi, *Eur. J. Org. Chem.* **2001**, 1441.

Alcoholysis of prochiral glutaric anhydrides under the usual conditions gives, with moderate selectivities, the monoesters **3–8**.

Lipase-catalyzed enantiomer-differentiating hydrolysis of racemic phenyl benzyl oxazolin-2-one in aqueous solution in combination with an uncatalyzed *in situ* racemization of the unchanged enantiomer of the heterocyclic system, with two different lipases, gives access to D- and L-*N*-benzoyl-phenylalanine **9** and *ent*–**9**, respectively. Enantiomer-differentiating alcoholysis and *in situ* racemization in organic solvents in the presence or absence of added water under the catalysis of lipase can in some cases furnish amino acid derivatives (**10–18**) with good selectivities and yields.

During alcoholysis of racemic substituted glutaric acid anhydride one is faced with regio- and enantioselectivity. These two processes may not cooperate in a matching sense. Despite this fact, the monoalkyl glutarates **19–21** have been obtained with moderate to good enantiomeric excess by lipase-catalyzed alcoholysis of the corresponding anhydrides in the presence of *Candida antarctica* B lipase with 2-methylpropanol.

Alcoholysis of alkyl carboxylates is due to the competition of the two alcohols characterized by reversibility and associated with low conversion and poor enantioselectivity. The alcoholysis of vinyl carboxylates in the presence of *Candida antarctica* B lipase with *n*-hexanol as demonstrated for **22** can be regarded as an alternative in order to overcome these difficulties.

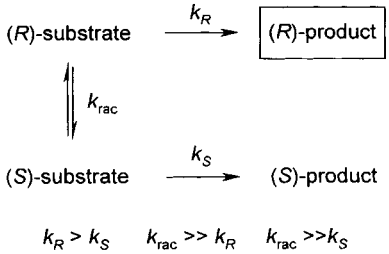
Esterification of carboxylic acids (**25**, **26**) (Table 11.1-23) in the presence of an orthoester as water-trapping agent may have advantages.

11.1.2.1.2 Dynamic Kinetic Resolution

The success of an enzyme-catalyzed kinetic resolution is limited by the maximum chemical yield of 50% for each enantiomer. However, this drawback can be overcome by a process called dynamic kinetic resolution. The key idea of this principle is to racemize the slow reacting enantiomer continuously reproducing the faster one. In an ideal case at the end of the conversion one enantiomer is formed in 100% yield with 100% of enantiomeric excess[135–137]. The kinetic requirements for a dynamic kinetic resolution are shown in Scheme 11.1-16[8b].

The *in situ* racemization can be achieved by different means either spontaneously or catalytically. Due to their chemical properties certain substrates may racemize spontaneously under the reaction conditions. Useful catalysts could be ordinary chemicals such as bases, transition metal complexes and in theory another type of biocatalyst. Having identified a suitable enzyme promoting the enantiomer-differentiating process by hydrolysis or alcoholysis of a carboxylic ester or by acylation of an alcohol one has to find the appropriate racemizing catalyst. Lipase and catalyst must tolerate each other; they must work under identical conditions. The product must be chemically and configurationally stable in the presence of the catalyst.

Table 11.1-24 lists lipase-catalyzed dynamic kinetic resolutions by different means. 4-Substituted oxazolin-5-ones racemize spontaneously by hydrolysis or alcoholysis caused by enolization to yield amino acid derivatives as outlined in the transformations (**1**), (**2**) and (**3**). Triethylamine may promote this type of transformations as



Scheme 11.1-16. Dynamic kinetic resolution.

Table 11.1-24. Lipase-catalyzed dynamic kinetic resolution (PCL *Pseudomonas cepacia* lipase, PPL pig pancreatic lipase, ANL *Aspergillus niger* lipase, MML *Mucor miehei* lipase, CAL-B *Candida antarctica* B lipase, PSL *Pseudomonas* sp. lipase, PFL *Pseudomoas fluorescens* lipase, CAL *Candida antarctica* lipase, not specified).

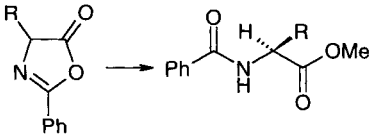
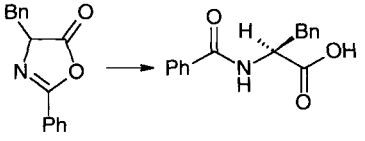
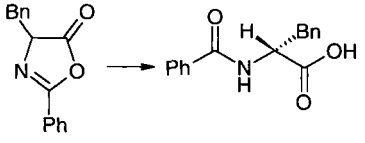
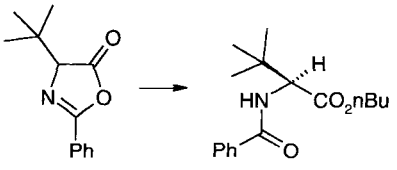
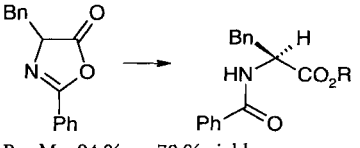
Reaction	Type	Racemization	
	alcoholysis	spontaneous	(1) [1]
R = *i*-Pr, Me_2CHCH_2, $MeSCH_2CH_2$, 2-Naphthyl-CH_2, 4-$MePhCH_2$, Ph, $PhCH_2$, $Ph(CH_2)_2$, $Ph(CH_2)_3$ R = Me_2CHCH_2: 78 % ee, 82 % yield, PCL, MeOH; 90 % ee, 85 % yield, MeOH+H_2O R = $Ph(CH_2)_3$: 84 % ee, 91 % yield, PCL, MeOH 95 % ee, 76 % yield, MeOH+H_2O			
	hydrolysis	spontaneous	(2) [2]
99 % ee, –, PPL			
	hydrolysis	spontaneous	(3) [2]
99 % ee, –, ANL			
	alcoholysis	NEt_3	(4) [3]
>99 % ee, 67 % yield, MML, *n*-BuOH, toluene			
	alcoholysis	NEt_3	(5) [4]
R = Me: 94 % ee, 79 % yield R = Et: 97 % ee, 82 % yield R = *n*-Pr: 97 % ee, 83 % yield			

Table 11.1-24. (cont.).

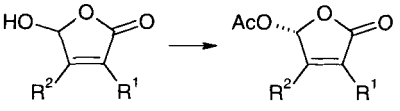
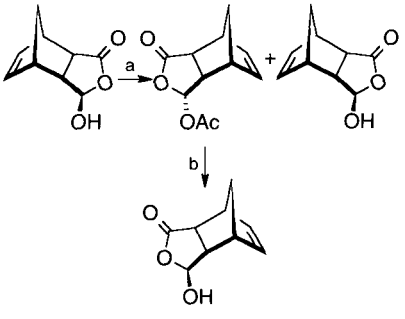
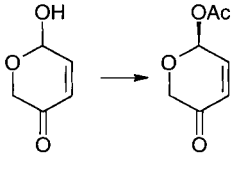
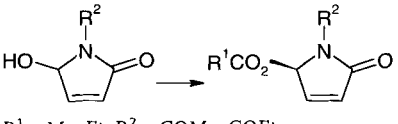
Reaction	Type	Racemization	
R = *n*-Bu: 95 % ee, 81 % yield all CAL-B, toluene R = Me: 97 % ee, 71 % yield, CAL-B, THF R = Me: 98 % ee, 88 % yield, CAL-B, MeCN			
 $R^1 = R^2 = H$; $R^1 = R^2 = Me$; $R^1 = Me$, $R^2 = H$; $R^1 = H$, $R^2 = Me$ 78–86 % ee, 100 % yield, PCL, vinyl acetate	acylation	spontaneous	(6) [5]
 a: acetate >90 % ee, 45 % yield, alcohol >90 % ee. 39 % yield, PCL, vinyl acetate b: 98 % ee, 100 % yield, PCL, *n*-BuOH, CH_2Cl_2	a: acylation b: alcoholysis	 spontaneous epimerization	(7) [6]
 76 % ee, >99 % conversion, PCL, vinyl acetate 79 % ee, 93 conversion, PSL, vinyl acetate	acylation	spontaneous	(8) [7]
 R^1 = Me, Et, R^2 = COMe, COEt >99 % ee, 100 % yield, CAL-B, *n*-hexane/CH_2Cl_2	acylation	spontaneous at >40 °C	(9) [8]

Table 11.1-24. (cont.).

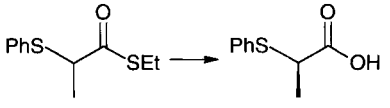
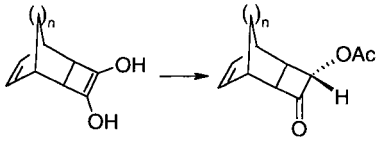
Reaction	Type	Racemization	
 R = 3-PhOC$_6$H$_4$, Ph, 4-Cl-C$_6$H$_4$, 3,4-OCH$_2$O-C$_6$H$_3$, 2-naphthyl, 1-naphthyl 70–96 % ee, 64–88 % yield, PSL, vinyl acetate	acylation	basic ion-exchange resin (**10**) [9]	
 R^1 = CO$_2$Me, BnOCH$_2$, AcOCH$_2$ R^2 = *n*-Bu, Et$_3$SiO(CH$_2$)$_2$, *i*-Pr, *n*-Octyl 87–>95 % ee, 63–87 % yield, PFL, vinyl acetate, *t*-BuOMe	acylation	silica gel	(**11**) [10]
 96 % ee, >99 % conversion, PCL, H$_2$O/toluene	hydrolysis	N(*n*-C$_8$H$_{17}$)$_3$	(**12**) [11]
 n = 1: 97 % ee, 75 % yield, PCL, vinyl acetate n = 2: 99 % ee, 67 % yield, PCL, vinyl acetate	acylation	NEt$_3$	(**13**) [12]
 R = *n*-Pr, R^1 = Me, R^2 = *n*-C$_6$H$_{13}$ 94 % ee, 100 % yield, PPL, vinyl propionate	acylation	Mitsunobu inversion	(**14**) [13]
R = Me, R^1 = Me, R^2 = Ph 97 % ee, 97 % yield, PCL, vinyl acetate	acylation		
R = *n*-Pr, R^1 = Ph, R^2 = CH$_2$NCOnPr, 97 % ee, 100 % yield, PCL, *n*-PrOH	alcoholysis of the (*S*)-acetate		
R = Me, R^1 = Aryl, R^2 = CN 61–97 % ee, 68–92 % yield, PCL, *n*-PrOH	alcoholysis of the (*S*)-acetate		

Table 11.1-24. (cont.).

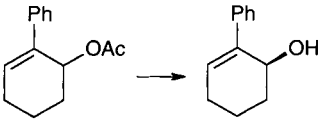
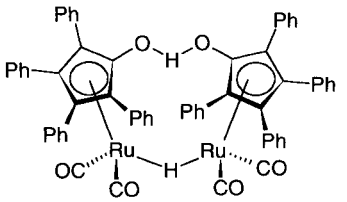
Reaction	Type	Racemization	
 96 % ee, 81 % yield, PFL	hydrolysis	$PdCl_2(MeCN)_2$	(15) [14]
 80 % ee, 76 % conversion, PFL, vinyl acetate, *ortho*-phenanthroline, PhCOMe, KOH 98 % ee, 60 % conversion, PFL, vinyl acetate, *ortho*-phenanthroline, PhCOMe	acylation	$[Rh(cod)Cl]_2$ $Rh_2(OAc)_4$	(16) [15]
   **A** >99 % ee, 92 % yield, CAL-B, 4-Cl-C_6H_4OAc, PhCOMe, *t*-BuOH	acylation	**A**	(17) [16]
 R^1 = 4-Br-C_6H_4, R^2 = Me; R^1 = 1-Naphthyl, R^2 = Me; R^1 = 2-naphthyl, R^2 = Me; R^1 = $PhOCH_2$, R^2 = Me; R^1 = *c*-C_6H_{11}, R^2 = Me; R^1 = *n*-C_6H_{13}, R^2 = Me; R^1 = Ph, R^2 = Et >98 % ee, 65–80 % yield, CAL-B, 4-Cl-C_6H_4OAc, PhCOMe, *t*-BuOH R^1 = 4-OMe-C_6H_4, R^2 = Me 91 % ee, 60 % yield, CAL-B, 4-Cl-C_6H_4 acetate, PhCOMe, *t*-BuOH R^1 = $PhOCH_2$, R^2 = CH_2Cl 79 % ee, 68 % yield, CAL-B, 4-Cl-C_6H_4OAc, PhCOMe, *t*-BuOH	acylation	**A**, cf (17)	(18) [17]

Table 11.1-24. (cont.).

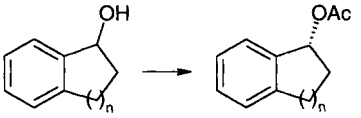
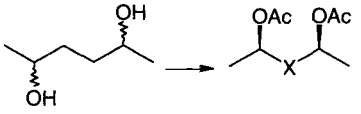
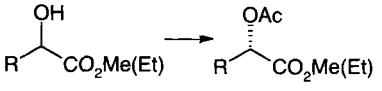
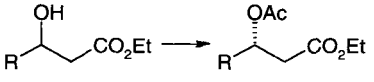
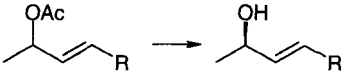
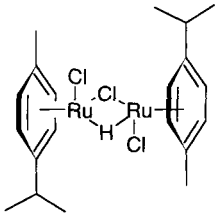
Reaction	Type	Racemization	
 n = 1, 2: >99 % ee, 65 and 77 % yield, CAL-B, 4-Cl-C_6H_4OAc, PhCOMe, *t*-BuOH	acylation	A, cf (17)	(19) [17]
 *rac/meso*-mixture (~50:50) → (*R,R/meso*) (76:26–100:0) (38:62 for X = CH_2) X = CH_2, $(CH_2)_2$, $(CH_2)_3$, (*E*)-CH=CH, 1,3-C_6H_4, 1,4-C_6H_4, 2,6-pyridylene, $CH_2N(Bn)CH_2$ >96–>99 % ee, 47–90 % yield, CAL-B, 4-Cl-C_6H_4OAc, toluene	acylation	A, cf (17)	(20) [17, 18]
 R = Ph, 4-OMe-C_6H_4, 4-Br-C_6H_4, *c*-C_6H_{11}, *n*-Bu 80–98 % ee, 60–80 % yield, PCL, 4-Cl-C_6H_4OAc, cyclohexane	acylation	A, cf (17)	(21) [19]
 R = Ph: 95 % ee, 73 % yield R = 4-OMe-C_6H_4: 99 % ee, 69 % yield R = $PhCH_2$: 96 % ee, 75 % yield R = *c*-C_6H_{11}: 70 % ee, 71 % yield all PCL, 4-Cl-C_6H_4OAc, cyclohexane	acylation	A, cf (17)	(22) [20]
 R = Ph, 4-Cl-C_6H_4, 4-Me-C_6H_4, 2-Furyl, 1-Naphthyl 97–>99 % ee, 70–87 % ee, CAL or PCL, *i*-PrOH, THF	alcoholysis	$Pd(PPh_3)_4dppf$	(23) [21]

Table 11.1-24. (cont.).

Reaction	Type	Racemization	
	acylation	**B**, NEt_3	(24) [22]
 **B**			
R = Ph, 4-Cl-C_6H_4, 4-Me-C_6H_4, 4-OMe-C_6H_4, 2-Furyl, 1-naphthyl, *c*-C_6H_{11}, *t*-Bu, CH_2-$CHMe_2$, *i*-Pr, *n*-Pr 95–>99 % ee, 84–91 % yield, PCL, 4-Cl-C_6H_4OAc, CH_2Cl_2			
	acylation	racemase	(25) [23]
>98 % ee, 80 % yield, 1. PSL, vinyl acetate, 2. mandelate racemase			

1 J. Z. Crich, R. Brieva, P. Marquart, R.-L- Gu, S. Flemming, C. J. Sih, *J. Org. Chem.* **1993**, *58*, 3252.
2 R.-L. Gu, I.-S. Lee, C. J. Sih, *Tetrahedron Lett.* **1992**, *33*, 1953.
3 N. J. Turner, J. R. Winterman, R. McCague, J. S. Parratt, S. J. C. Taylor, *Tetrahedron Lett.* **1995**, *36*, 1113.
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16 A. L. E. Larsson, B. A. Persson, J.-E. Bäckvall, *Angew. Chem.* **1997**, *109*, 1256; *Angew. Chem. Int. Ed. Engl.* **1997**, *36*, 1211.
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18 B. A. Persson, F. F. Huerta, J.-E. Bäckvall, *J. Org. Chem.* **1999**, *64*, 5237.
19 F. F. Huerta, Y. R. S. Laxmi, J.-E. Bäckvall, *Org. Letters* **2000**, *2*, 1037.
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21 Y. K. Choi, J. H. Suh, D. Lee, I. T. Lim, J. Y. Jung, M.-J. Kim, *J. Org. Chem.* **1999**, *64*, 8423.
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shown for (4) and (5). The hemiacetal and hemiaminal derivatives formed by the reactions (6)–(9) have been obtained by lipase-catalyzed acylation of the configurationally unstable hemiacetals or-aminal, respectively, or by alcoholysis of an acylated hemiacetal as shown for (7). The latter case is not a dynamic kinetic resolution but a normal kinetic resolution (acylation) followed by a spontaneous epimerization by lipase-catalyzed alcoholysis. Remarkably, the non-acylated hemiaminals are configurationally stable at temperatures below 40 °C and dynamic kinetic resolution proceeds according to reaction (9) at higher temperatures. Cyanohydrins are unstable under basic conditions regenerating the starting materials aldehyde and hydrogen cyanide. This property was used to prepare enantiomerically enriched cyanohydrin acetates according to transformation (10) by reaction of the corresponding aldehydes with acetone cyanohydrin in the presence of vinyl acetate, *Pseudomonas* sp. lipase and a strong basic ion-exchange resin as racemizing catalysts. The latter transformation demonstrates that lipase-catalyzed-acylation of one of the cyanohydrin enantiomers is faster than conversion of the cyanohydrin into aldehyde and hydrogen cyanide.

The 2-acetoxysulfides are obtained according reaction (11) by in situ formation of the configurationally unstable hemithioacetals and subsequent lipase-catalyzed acylation. Racemization of the hemithioacetals was achieved by silica gel. The 2-phenylthiocarboxylic acid was formed by hydrolysis of the corresponding thioester in the presence of trioctylamine [reaction (12)].

The formation of the 2-acetoxy ketone in the reaction (13) was achieved by shifting an enediol-hydroxyketone equilibrium with triethylamine.

The formation of the enantiomerically enriched or pure esters by reaction (14) was not a result of a dynamic process but a one-pot two-step procedure consisting of lipase-catalyzed resolution and a subsequent inversion of the slow reacting enantiomeric alcohol by Mitsunobu reaction.

Enantiomerically pure allylic alcohols were prepared by a dynamic kinetic resolution in the transformations (15) and (23) by lipase-catalyzed hydrolysis or alcoholysis of the corresponding acetates in the presence of palladium complexes racemizing the slow reacting enantiomeric acetates. 1-Phenylethanol was converted in an acylation process into the corresponding acetate in reaction (16) by two different types of rhodium catalysts. On the other hand, 1-phenylethanol and a variety of the further secondary alcohols were obtained with high enantiomeric excess under in situ racemization with the ruthenium catalyst **A** with very high efficiency [reactions (17)–(19)]. Moreover, this catalyst was used also for the racemization/epimerization procedure (20) converting an approximately 1 : 1 mixture of *racemic/meso*-diols into the corresponding enantiomeric diacetates under consumption of the *meso*-diol as well as for the dynamic kinetic resolution of 2- and 3-hydroxy carboxylic esters as shown in the reactions (21) and (22), respectively, under acylation conditions. The ruthenium catalyst was not compatible with vinyl acetate and therefore, 4-chlorophenyl acetate was found to be the acylating agent of choice. A redox process can explain the racemization of the slow reacting enantiomeric alcohol.

Another ruthenium catalyst was used for the dynamic kinetic resolution of allylic alcohols [reaction (24)] by acylation yielding allylic acetates. Again a redox process should be responsible for the racemization.

Table 11.1-25. The beneficial influence of additives on lipase-catalyzed enantiomer- and enantiotopos-differentiating reactions (PCL *Pseudomonas cepacia* lipase, CCL *Candida cylindracea* lipase, CAL *Candida antarctica* lipase, not specified, LIP *Pseudomonas* sp. lipase-Toyobo, PFL *Pseudomoas fluorescens* lipase, CAL-B *Candida antarctica* B lipase, MML *Mucor miehei* lipase).

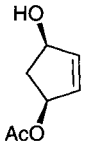
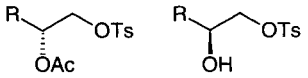
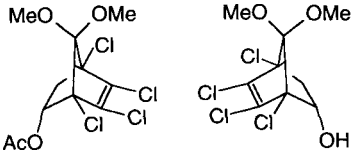
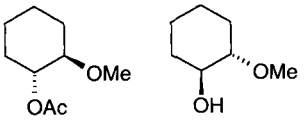
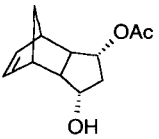
Product(s)	Additive	Influence of the additive	
			**1** [1]
pancreatin, $AcOCH_2CCl_3$, THF no reaction without NEt_3	NEt_3	reaction rate	
pancreatin, vinyl acetate, no further solvent from 72 to >99 % ee	NEt_3	selectivity	
 PCL, vinyl acetate, *t*-BuOMe R = Vinyl: from $E = 50$ to >200 R = CH_2Cl: from $E = 39$ to > 200 R = Et: from $E = 7$ to 60	NEt_3	selectivity	**2** [2]
 CCL, Ac_2O, toluene from $E = 19$ to 180 with 2,6-lutidine from $E = 19$ to 240 with $KHCO_3$	2,6-lutidine or $KHCO_3$	selectivity	**3** [3]
 CAL, vinyl acetate, cyclohexane	NEt_3	selectivity	**4** [4]
 LIP, vinyl acetate, THF from 10 d to 3 h	NEt_3	reaction rate	**5** [5]

Table 11.1-25. (cont.).

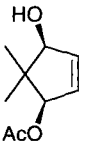
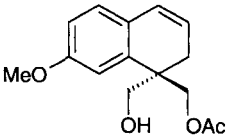
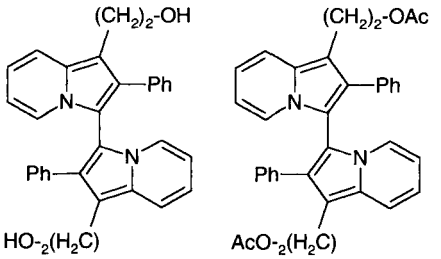
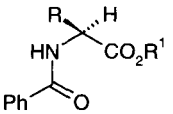
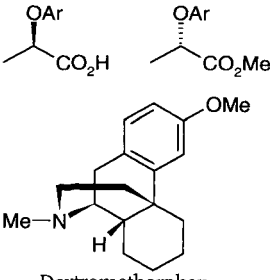
Product(s)	Additive	Influence of the additive	
 PFL on sawdust, vinyl acetate, THF	NEt_3	selectivity	**6** [6]
 PCL on Hyflo Super Cell®, vinyl acetate, *t*-BuOMe, from 85 to 93 % ee	NEt_3	selectivity	**7** [7]
 absolute configuration unknown CAL-B, vinyl acetate, THF	NEt_3	selectivity and reaction rate	**8** [8]
 MML or CAL-B, R^1OH, *n*-hexane or toluene R = *t*-Bu, R^1 = *n*-Bu: from 80 to >99 % ee	NEt_3	selectivity and reaction rate	**9** [9]
 Dextromethorphan	Dextromethorphan (DM) or its enantiomer Levomethorphan (LM)	selectivity and reaction rate	**10** [10]

Table 11.1-25. (cont.).

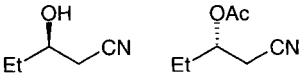
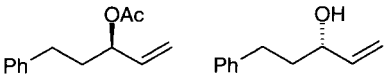
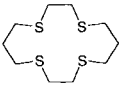
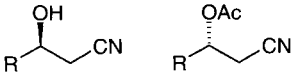
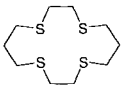
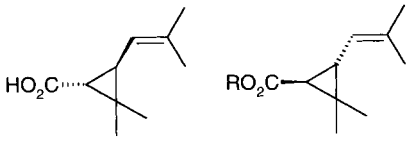
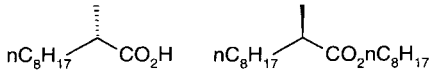
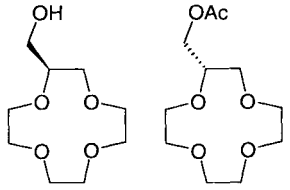
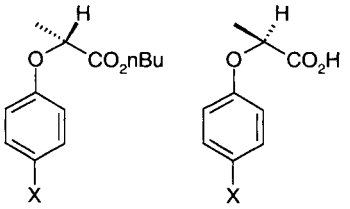
Product(s)	Additive	Influence of the additive	
CCL, phosphate buffer pH 7 Ar: 2-Me-4-Cl-C_6H_3: from $E = 1$ to 37 with DM and to $E = 81$ with LM			
 PCL, aqueous buffer, pH 7.2	Dextromethorphan (cf **10**) or (2*S*)-2-amino-4-methylthio-1-butanol	selectivity	**11** [11]
 PCL, vinyl alkanoates, *n*-hexane or *i*-Pr_2O		selectivity	**12** [12]
 PCL, acetone/water	 and further crown ethers	selectivity and reaction rate	**13** [13]
 CCL, aqueous buffer pH 7.2	Triton X-100	selectivity	**14** [14]
 CCL, aqueous buffer pH 8	$CaCl_2$	selectivity	**15** [15]
 PCL, H_2O	NaCl	selectivity	**16** [16]

Table 11.1-25. (cont.).

Product(s)	Additive	Influence of the additive	
 CCL, *n*-BuOH, *i*-Pr_2O X = Et: from E = 3.8 to 201 X = CF_3: from E = 1.3 to 56	aqueous LiCl	selectivity and reaction rate	17 [17]

1 a) F. Theil, S. Ballschuh, H. Schick, M. Haupt, B. Häfner, S. Schwarz, *Synthesis* **1988**, 540.; b) F. Theil, H. Schick, M. A. Lapitskaya, K. K. Pivnitsky, *Liebigs Ann. Chem.* **1991**, 195.
2 N. W. Boaz, R. L. Zimmerman, *Tetrahedron: Asymmetry* **1994**, *5*, 153.
3 B. Berger, C. G. Rabiller, K. Königsberger, K. Faber, H. Giengl, *Tetrahedron: Asymmetry* **1990**, *1*, 541.
4 P. Stead, H. Marley, M. Mahmoudian, G. Webb, D. Noble, Y. T. Ip, E. Piga, T. Rossi, S. M. Roberts, M. J. Dawson, *Tetrahedron: Asymmetry* **1996**, *7*, 2247.
5 a) T. Sugahara, K. Ogasawara, *Synlett* **1996**, 319; b) T. Sugahara, Y. Kuroyanagi, K. Ogasawara, *Synthesis* **1996**, 1101.
6 W. Kreiser, A. Wiggermann, A. Krief, D. Swinnen, *Tetrahedron Lett.* **1996**, *37*, 7119.
7 A. Fadel, P. Arzel, *Tetrahedron: Asymmetry* **1997**, *8*, 283.
8 F. Theil, H. Sonnenschein, T. Kreher, *Tetrahedron: Asymmetry* **1996**, *7*, 3365.
9 a) N. J. Turner, J. R. Winterman, R. McCague, J. S. Parratt, S. J. Taylor, *Tetrahedron Lett.* **1995**, *36*, 1113; b) M.-C. Parker, S. A. Brown, L. Robertson, N. J. Turner, *Chem. Commun.* **1998**, 2247; c) S. A. Brown, M.-C. Parker, N. A. Turner, *Tetrahedron: Asymmetry* **2000**, *11*, 1687.
10 Z.-W. Guo, C. J. Sih, *J. Am. Chem. Soc.* **1989**, *111*, 6836.
11 T. Itoh, E. Ohira, Y. Takaki, S. Nishiyama, K. Nakamura, *Bull. Chem. Soc. Jpn.* **1991**, *64*, 624.
12 a) Y. Takaki, J. Teramoto, H. Kihara, T. Itoh, H. Tsukube, *Tetrahedron Lett.* **1996**, *37*, 4991; b) Y. Takaki, R. Ino, H. Kihara, T. Itoh, H. Tsukube, *Chem. Lett.* **1997**, 1247.
13 a) T. Itoh, Y. Takaki, T. Murakami, Y. Hiyama, H. Tsukube, *J. Org. Chem.* **1996**, 61, 2158; b) T. Itoh, K. Mitsukara, W. Kanphai, Y. Takaki, J. Teramoto, H. Kihara, H. Tsukube, *J. Org. Chem.* **1997**, *62*, 9165.
14 A. Bashkar Rao, H. Rehman, B. Krishnakumari, J. S. Yadav, *Tetrahedron Lett.* **1994**, *35*, 2611.
15 E. Holmberg, M. Holmquist, E. Hedenström, P. Berglund, T. Norin, H.-E. Högberg, K. Hult, *Appl. Microbiol. Biotechnol.* **1991**, *35*, 572.
16 a) H. Tsukube, A. Betchaku, Y. Hiyama, T. Itoh, *J. Chem. Soc., Chem. Commun.* **1992**, 1751; b) H. Tsukube, A. Betchaku, Y. Hiyama, T. Itoh, *J. Org. Chem.* **1994**, *59*, 7014.
17 T. Okamoto, S. Ueji, *Chem. Commun.* **1999**, 939.

In reaction (**25**) racemization was realized by madelate racemase. However, this transformation is still a process carried out in two batches and therefore, not a dynamic kinetic resolution but certainly the starting point for further investigations by combining a lipase- and a second enzyme-catalyzed reaction in order to perform real dynamic kinetic resolution.

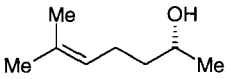
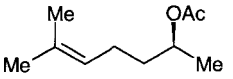
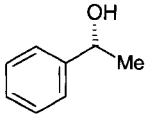
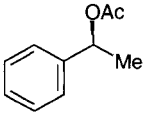
11.1.2.1.3 Enhancement of Selectivity and Reactivity of Lipases by Additives

It has been shown that additives have a great potential for fine-tuning the reaction conditions for lipase-catalyzed reactions[133]. Certain additives such as tertiary amines, thiacrown ethers or inorganic salts may increase the selectivity and/or reaction rate. The reason for these effects are little understood and only a few systematic investigations have been undertaken. From a synthetic chemist's point of

view treatment of the reaction mixture with an additive is a convenient way to improve the outcome of the reaction.

Table 11.1-25 list examples in which certain additives have an unambiguous beneficial influence on selectivity and/or reaction rate. The enantiomerically enriched or pure compounds **1–9** have been prepared under the influence of mainly triethylamine or other bases. In case of **1** for the acetylation with 2,2,2-trichloroethyl acetate there was no reaction without triethylamine. For the formation of **5** the reaction time was shortened dramatically from ten days to three hours for 100% of conversion. In most cases there is no rationale for the effects of bases except the formation of ion-pairs between the added bases and traces of acids present in the reaction mixture. Only for the synthesis of **9** a systematic investigations demonstrates that triethylamine besides its racemizing properties (cf. Table 11.1-24) has a significant influence on the water activity of the reacting mixture[138]. In other cases, triethylamine has been used as an additive without comparing its influence with the results in its absence[133].

Table 11.1-26. Subtilisin-catalyzed acylation of racemic alcohols in organic solvents.

		[1]
R = Et, v_S/v_R = 3.9, dioxane		1
R = *n*Bu, E = 28		2
R = $(CH_2)_2CH{=}CMe_2$, E = 11		3
R = *n*Hex, v_S/v_R = 100, dioxane		4
R = *n*Dec, E = 100		5
R = Ph, v_S/v_R = 50, dioxane		6
R = 2-naphthyl, v_S/v_R = 58, dioxane		7
vinylbutyrate, vinylacetate		
	8a [2]	 8b [2]
≥98% ee		54% ee
64% conversion		64% conversion
vinylacetate		
	9a [2]	 9b [2]
40% ee		92% ee
30% conversion		30% conversion
vinylacetate		

1 P. A. Fitzpatrick, A. M. Klibanov, *J. Am. Chem. Soc.* **1991**, *131*, 3166.
2 Y.-F. Wang, K. Yakovlevsky, B. Zhang, A. L. Margolin, *J. Org. Chem.* **1997**, *62*, 3488.

The enantiomerically pure tertiary bases dextro- and levomethorphan were used for the preparation of 2-aryloxy propionic acid derivatives **10** by increasing the selectivity dramatically based on the enantioselective inhibition of the slow reacting enantiomer.

The kinetic resolutions yielding the enantiomers **12** and **13** were conducted in the presence of thiacrown and some further crown ethers. Inorganic salts as shown for **15–17** were suitable modulators of selectivity and/or reaction rate. Particularly, in case of **17** a strong increase of the enantiomer-selectivity was found by the addition of a defined amount of aqueous lithium chloride solution to the reaction mixture. The E value was increased by factors between ten to fifty depending on the substrate structure.

11.1.1.2.2 **Subtilisin**

A beneficial feature of subtilisin, and in particular subtilisin-CLECs, is their high catalytic activity in polar and non-polar organic solvents, allowing for transesterifications of alcohols in the presence of small amounts of water. Transesterifications catalyzed by subtilisin were mostly done with vinyl acetate. Apparently, the acetaldehyde formed during transesterification is not harmful to the enzyme as it is in the case of some lipases and pig liver esterase. Although resolution of such alcohols either through hydrolysis of the corresponding esters or transesterification is the domain of lipase, in some cases useful selectivities were achieved with subtilisin (**1–9**) (Table 11.1-26).

11.1.1.2.3 **Pig Liver Esterase**

Pig liver esterase-catalyzed enantioselective acylation of prochiral or racemic alcohols in organic solvents has not nearly gained the importance of the lipase-catalyzed acylation method. This is due to the fact that pig liver esterase shows only very low activity in organic solvents. The esterase differs considerably in this respect from lipases and subtilisin, which are both highly active in organic media. Attempts to confer activity to pig liver esterase in organic solvents by entrapment in water-filled porous supports[139], covalent attachment of MPEG residues[140, 141], immobilization on carrageenan[142] and Eupergit[143, 144], or entrapment in polymers[145] were met with various degrees of success. It was found, however, that colyophylization of pig liver esterase with MPEG significantly enhances the activity and stability of the enzyme in organic solvents of low to medium polarity and low water content[66, 146, 147]. The colyophilizate of pig liver esterase and MPEG was successfully applied to the kinetic resolution of racemic glycerol derivatives through acylation with vinyl and isopropenyl esters in toluene containing less than 1 % water (**1**, **3**, and **5**) (Table 11.1-27). Medium selectivities were recorded for alcohols having a primary hydroxyl group, and a high selectivity was found in the case of the glycerol derivative with a secondary hydroxyl group. Pig liver esterase-catalyzed hydrolysis of the corresponding racemic esters in water occurred with lower selectivities (**2**, **4** and **6**). A number of functionalized secondary alcohols have been resolved with high

Table 11.1-27. Pig liver esterase-catalyzed acylation of racemic alcohols in organic solvents.

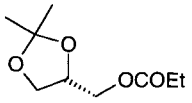
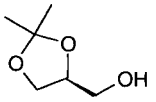
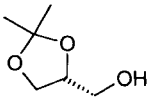
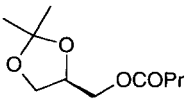
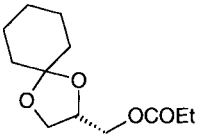
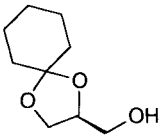
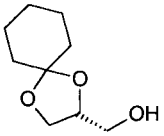
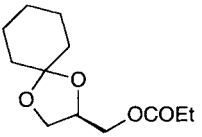
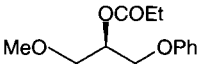
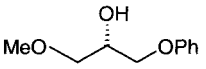
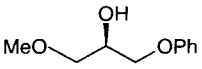
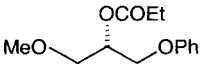
	**1a** [1, 2] E = 24 vinyl propionate, toluene		**1b** [1, 2]
	**2a** [1, 2] E = 3–5 hydrolysis in water		**2b** [1, 2]
	**3a** [1, 2] E = 30 vinyl propionate, toluene		**3b** [1, 2]
	**4a** [1, 2] E = Z hydrolysis in water		**4b** [1, 2]
	**5a** [1, 2] E >100 vinyl propionate, toluene		**5b** [1, 2]
	**6a** [1, 2] E ≈ 1 hydrolysis in water		**6b** [1, 2]

Table 11.1-27. (cont.).

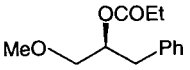
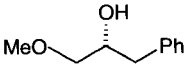
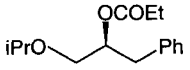
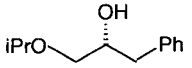
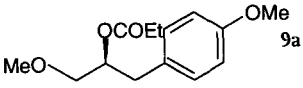
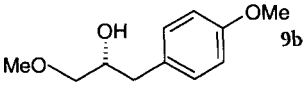
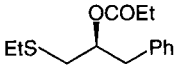
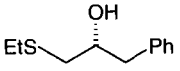
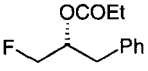
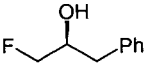
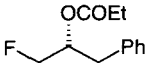
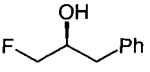
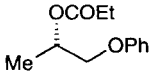
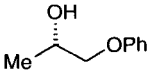
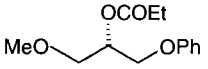
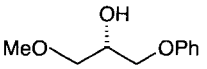
	**7a** [1, 2] E = 100 vinyl propionate, toluene		**7b** [1, 2]
	**8a** [1, 2] E >100 vinyl propionate, toluene		**8b** [1, 2]
	**9a** [1, 2] E = 50 vinyl propionate, octane		**9b** [1, 2]
	**10a** [1, 2] E >100 vinyl propionate, toluene		**10b** [1, 2]
	**11a** [1, 2] E >100 vinyl propionate, toluene		**11b** [1, 2]
	**12a** [1, 2] E = 50 vinyl propionate, toluene		**12b** [1, 2]
	**13a** [1, 2] E–100 vinyl propionate, toluene		**13b** [1, 2]

Table 11.1-27. (cont.).

	**14a** [1, 2] E >100 vinyl propionate, toluene		**14b** [1, 2]

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selectivity by pig liver esterase-catalyzed acylation with vinyl propionate in toluene. As in the case of lipases, a competing pig liver esterase-catalyzed hydrolysis of vinyl propionate and a partial deactivation of the enzyme by the acetaldehyde formed in transesterification had been observed. Critical to the activity and selectivity of pig liver esterase in the presence of MPEG in organic solvents is the water content of the system, which should be lower than 1%. In general, the activity of pig liver esterase in the presence of MPEG in organic solvents is lower than that of lipases and subtilisin under comparable conditions. The colyophilizate of pig liver esterase and MPEG can be recovered from organic media with a minor loss of activity through a spontaneous immobilization on an ultrafiltration membrane placed in the reaction mixture[66].

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11.2
Hydrolysis of Epoxides

Kurt Faber and Romano V. A. Orru

Chiral epoxides and 1,2-diols, which are central building blocks for the asymmetric synthesis of bioactive compounds, can be obtained via the asymmetric hydrolysis of epoxides using enzymes – i.e. epoxide hydrolases (EHs) [EC 3.3.2.X]. Enzymes from mammalian sources – such as rat liver tissue – have been investigated in great detail for several decades during detoxification studies[1]; however, their application for biotransformations on a preparative scale was hampered because of the limited supply of these enzymes, and, as a consequence, the examples reported rarely surpass the millimolar range[2, 3]. During the past few years, highly selective epoxide hydrolases were identified from a wide range of microbial sources, which allows for an (almost) unlimited supply of these enzymes for preparative-scale applications. These valuable biocatalysts have recently gained considerable attention, and their scope and limitations have been reviewed[4–8]. Microbial epoxide hydrolases were found to be more abundant than previously expected, and numerous sources, predominantly among bacteria, fungi and (red) yeasts are known to date.

The mechanism of enzymatic hydrolysis of epoxides can be compared to that of base-catalysis, i.e. it resembles an S_N2-type opening of the epoxide by the nucleophile (i.e. water), which leads to the formation of the corresponding *trans*-configurated 1,2-diol. Any chiral center present in the substrate oxirane can be "recognized", thus effecting kinetic resolution or asymmetrization of racemic or *meso*-epoxides, respectively. The data available to date indicate that the enantioselectivities of enzymes from certain microbial sources can be correlated to the substitutional pattern of various types of substrates: red yeasts (*Rhodotorula* or *Rhodosporidium* sp.) give best enantioselectivities with monosubstituted oxiranes; fungal cells (e.g. from *Aspergillus* and *Beauveria* sp.) are best suited for styrene oxide-type substrates, whereas bacterial enzymes (in particular from *Actinomycetes* such as *Rhodococcus* and *Nocardia* sp.) are the biocatalysts of choice for more highly substituted 2,2- and 2,3-substituted epoxides. In order to overcome the disadvantage of the classic kinetic resolution pattern, i.e. the formation of two enantiomers in each 50% yield, various deracemization methods based on chemo-enzymatic or purely enzymatic protocols have been developed. The latter led to the highly desirable formation of a single stereoisomer of the diol in 100% theoretical yield. The synthetic potential of epoxide hydrolases for asymmetric synthesis has been proven by the preparation of a number of bioactive compounds.

Chiral epoxides and vicinal diols (employed as their corresponding cyclic sulfate or sulfite esters as reactive intermediates) are extensively employed high-value intermediates in the synthesis of chiral compounds because of their ability to react with a broad variety of nucleophiles (Figs. 11.2-1 and 11.2-2). In recent years, extensive efforts have been devoted to the development of chemo-catalytic methods for their production[9, 10]. Thus, the Sharpless methods allowing for the asymmetric epoxida-

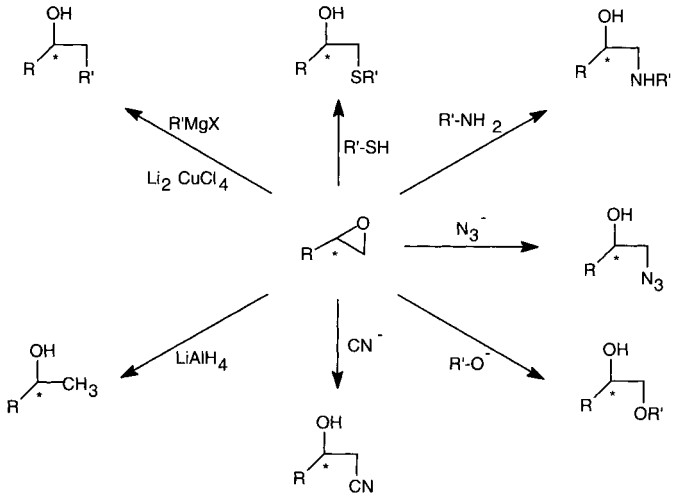

Figure 11.2-1. Reaction of epoxides with nucleophiles.

tion of allylic alcohols [11] and the asymmetric dihydroxylation of alkenes [10] are now widely applied reliable procedures. In addition, asymmetric catalysts for the epoxidation of non-functionalized olefins [12–14] have been developed more recently. Although high stereoselectivity has been achieved for the epoxidation of *cis*-alkenes, the results obtained with *trans*- and terminal olefins were less satisfactory using the latter method. More recently, two highly selective methods for the opening of terminal mono- and 2,2-disubstituted epoxides have been published. These methods are both based on a kinetic resolution using cobalt-salen complexes and water [15] or chromium-salen complexes and azide [16], respectively and have great potential in asymmetric synthesis [17].

On the other hand, a number of biocatalytic methods have been reported to provide a useful arsenal of methods as valuable alternatives to the above-mentioned techniques [18–23]. Prochiral or racemic synthetic precursors of epoxides, such as halohydrins, can be asymmetrized or resolved using hydrolytic enzymes [24, 25]. In particular, esterases and lipases have been used for such a enantioselective ester hydrolysis or esterification. This methodology is well developed, and high selectivities have been achieved in particular for esters of secondary alcohols, but it is impeded by the requirement of regioisomerically pure halohydrins. Furthermore, it is known that α-haloacid dehalogenases catalyze the S_N2-displacement of a halogen atom at the α-position of carboxylic acids with a hydroxy function. This process leads to the formation of the corresponding α-hydroxy acid with inversion of configuration [26]. However, α-haloacid dehalogenation incurs two drawbacks: (i) the instability of the substrates, particularly the α-bromoacids, in aqueous systems, and (ii) the limited substrate tolerance, as only short-chain haloacids are accepted [27]. Asymmetric biocatalytic reduction of α-keto-acids [28] using D- or L-lactate dehydrogenase or α-keto-alcohols [29] by glycerol dehydrogenase provides access to chiral α-hydroxyacids or 1,2-diols, which can be converted into the corresponding epoxides using conventional chemical methodology. Although excellent selectivities are generally

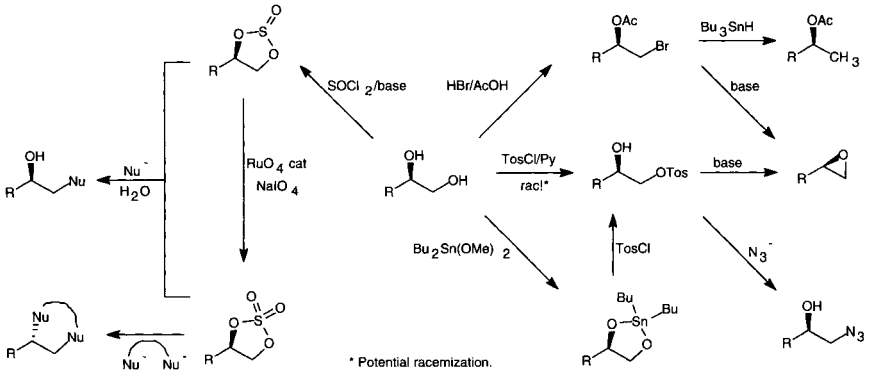

Figure 11.2-2. Syntheses from chiral 1,2-diols.

achieved, the need for the recycling of redox-cofactors such as NAD(P)H has restricted the number of applications. Likewise, biocatalytic asymmetric epoxidation of alkenes catalyzed by mono-oxygenases cannot be performed on a preparative scale with isolated enzymes, because of their complex nature and their dependence on a redox cofactor such as NAD(P)H. Thus, whole microbial cells have to be used instead. This method is not trivial and requires high bioengineering skills [30]. On the other hand, haloperoxidases are independent of nicotinamide-cofactors, as they produce hypohalous acid from H_2O_2 and halide, which in turn yields a halohydrin from an alkene. These enzymes are rare in Nature and exhibit usually low selectivities due to the fact that the formation of halohydrins can take place not only in the active site of the enzyme but also without enzyme catalysis [31]. Similar low selectivities have been observed with halohydrin epoxidases, which act like a "biogenic chiral base" by converting a halohydrin into the corresponding epoxide [32]. On the other hand, peroxidases, such as chloroperoxidase (CPO), are cofactor-independent and can be used in isolated form for the enzymatic epoxidation of alkenes [33–35].

An attractive alternative to the methods mentioned above is the use of cofactor-independent epoxide hydrolases, which are readily available from microbial sources in sufficient quantities.

11.2.1
Epoxide Hydrolases in Nature

In eukaryotes, microsomal and cytosolic epoxide hydrolases mainly play a key role in the detoxification of mutagenic, poisonous and carcinogenic epoxides [36, 37], which are formed by the action of P_{450}-dependent monooxygenases [38]. In addition, they are involved in the biosynthesis of hormones (e.g. leukotrienes). In plants, epoxide hydrolases are responsible for the generation of chiral aroma compounds and, in the biosynthesis of cutin, a wax-type polyester, which protects plants against microbial attack [39]. Insect epoxide hydrolases degrade juvenile hormones and pheromones bearing an oxirane moiety [3]. On the other hand, in microorganisms these enzymes

are multi-functional: (i) they can function as detoxifying agents, (ii) they can play a role in biosynthetic routes of complex (secondary) metabolites, or (iii) they may be crucial for the degradation of epoxides during the metabolism of alkenes and aromatics[40].

The degradation of aromatics in eukaryotes occurs *via* two different pathways (Fig. 11.2-3): (i) dioxygenase-catalyzed cycloaddition of molecular oxygen to the C=C bond yields a (putative) dioxetane species, which is then detoxified via reductive cleavage of the O-O bond yielding a physiologically more innocuous *cis*-1,2-diol; (ii) The formation of a highly reactive arene oxide via the introduction of a single O atom (from molecular oxygen) into the aromatic system is catalyzed by a mono-oxygenase. The latter epoxy species is further metabolized via hydrolysis catalyzed by an epoxide hydrolase to yield a *trans*-1,2-diol.

In lower organisms, alkenes can be metabolized in an analogous fashion, i.e. via an epoxide intermediate. In an analogous fashion, this intermediate is hydrolyzed to the corresponding 1,2-diol by an epoxide hydrolase. The latter product is degraded either by oxidation or by elimination of water under catalysis of a diol dehydratase, yielding an aldehyde[41]. Alternatively, such aldehydes are obtained via direct rearrangement of the epoxide catalyzed by an epoxide isomerase[42].

For a long time it was generally assumed that epoxide hydrolases are predominantly found in mammals[1, 2], although epoxide hydrolase activities had been detected in bacteria[43, 44] or fungi [45, 46] quite some years ago. This early view was certainly too simplistic, and enzymes of this type have now been detected in many bacteria[47–49], fungi[50], and red yeasts[51]. Moreover, epoxide hydrolase activity has been demonstrated in plants[52] and insects[53].

11.2.1.1
Isolation and Characterization of Epoxide Hydrolases

Several membrane-bound and soluble epoxide hydrolases from mammalian origin have been purified and (at least partially) sequenced. Some of them have also been cloned and overexpressed, which is the case for the soluble EH from rat liver which has been overexpressed in *Escherichia coli*[54, 55]. This enzyme (as well as its microsomal analog) was shown to share an amino acid sequence similarity to a region around the active center of a bacterial haloalkane dehalogenase[56], an enzyme with known three-dimensional structure that belongs to the α/β-hydrolase fold-family[57]. Rat soluble EH forms a dimer from two complete structural monomeric units, both possessing a distinct active site. The EH activity is known to be located close to the C-terminal unit, while the function of the N-terminal unit remains unknown[58].

To date, several epoxide hydrolases from microbial sources have been purified. For instance, from *Bacillus megaterium*[44], *Corynebacterium* sp.[59, 47] and *Pseudomonas* sp.[48, 60], but also from dematiaceous fungi such as *Ulocladium atrum* and *Zopfiella karachiensis*[61]. However, some of these were only partially purified, or their enantioselectivities were low or not investigated. In contrast, highly enantioselective epoxide hydrolases from *Rhodococcus* sp. NCIMB 11216[49] and *Nocardia* sp. EH1[62]

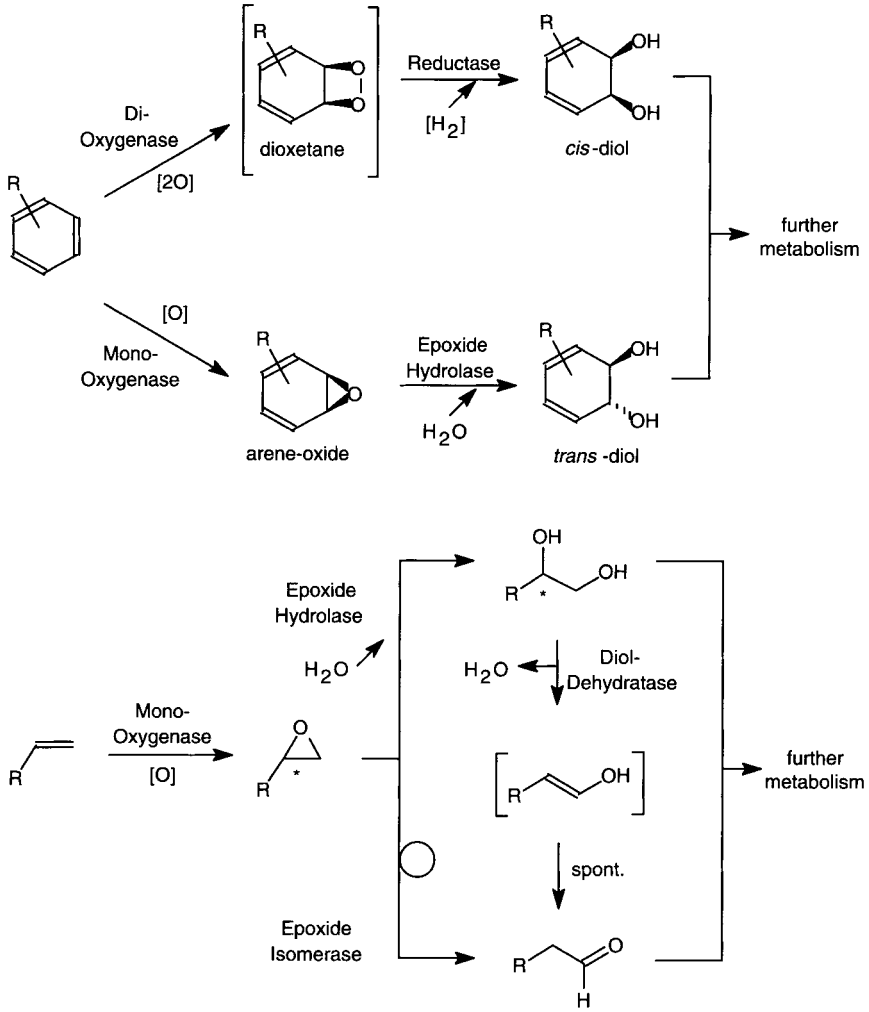

Figure 11.2-3. Involvement of epoxide hydrolases in the biodegradation of aromatics and alkenes.

were purified to homogeneity. Both (monomeric) proteins exhibit several common features: they are of similar size (≈ 34 kDa) and do not possess any metal ion or any UV-absorbing prosthetic group. The catalytic power of both enzymes was found to be in the same range (≈ 2000 μmol mg^{-1} h^{-1}), as was the optimum temperature (33–37 °C) and pH (7.5–9.0). The only notable difference between the two enzymes is the high instability of the *Nocardia* epoxide hydrolase (which completely loses its activity within hours, even when stored at −18 °C), whereas the *Rhodococcus* enzyme was shown to be relatively stable. It should be noted that the former enzyme could be stabilized by immobilization through ionic binding onto DEAE-cellulose. This resulted in a doubling of the activity (compared to the native enzyme) albeit at a

slight reduction in enantioselectivity[63]. The poor stability of the *Nocardia* enzyme and the fact that the N-terminus of the *Rhodococcus* epoxide hydrolase was unspecifically blocked precluded their N-terminal sequencing. In contrast, an epichlorohydrin-degrading epoxide hydrolase from *Agrobacterium radiobacter* AD1 could be isolated, characterized and sequenced after cloning and overexpression in *E. coli* BL21 (DE3)[64]. This enzyme showed an amino acid sequence similarity to eukaryotic epoxide hydrolases, haloalkane dehalogenase and bromoperoxidase, which indicated that it belongs to the α/β-hydrolase fold family. Most epoxide hydrolases from pro- or eukaryotic sources seem to belong to this group of enzymes. Another bacterial epoxide hydrolase from this family has been isolated from *Corynebacterium* sp. C12 when grown on cyclohexene oxide[65]. The purification to homogeneity was achieved in two steps. The enzyme is (partly) membrane bound and multimeric (probably tetrameric) which is in contrast to the enzymes described above. The subunit-size is ca. 32 kDa and amino acid sequence comparison showed that it is related to mammalian and plant (soluble) EH. Furthermore, it showed striking similarilties with the *Agrobacterium* enzyme, particularly around the catalytic site. Epoxide hydrolases from fungal sources were purified recently: an epoxide hydrolase from *Aspergillus niger* was purified to homogeneity[66] and appears to be a tetramer composed of four identical subunits of molecular mass 45 kDa. The N-terminus was blocked, the pH optimum lies at 7.0 and the temperature optimum at 40 °C. From red yeasts (*Rhodotorula glutinis*[67] and *Rhodosporidium toruloides* CBS0349[68]), two epoxide hydrolases have been purified. Both membrane-bound enzymes are medium-sized (45 and 54 kDa, respectively) and are structurally related to other microsomal epoxide hydrolases. They probably belong to the α/β-hydrolase fold family as well. An epoxide hydrolase with an unprecedented low molecular mass (only 17 kDa) was isolated from *Rhodococcus erythropolis* DCL14[69]. The cofactor-independent enzyme is efficiently induced when the microorganism was grown on monoterpenes, such as limonene, reflecting its special role in the limonene degradation pathway. The low molecular mass, the unusually broad pH-optimum (6.0–11.0) and the elevated optimum temperature (50 °C), together with the fact that the N-terminal amino acid sequence revealed no homology with any other protein, led to the conclusion that this protein does not belong to the α/β-hydrolase fold family.

11.2.1.2
Structure and Mechanism of Epoxide Hydrolases

The first X-ray structure of an epoxide hydrolase (from *Agrobacterium radiobacter* AD1) has been reported recently (Fig. 11.2-4)[70]. The nearly globular protein consists of a core-domain with typical features of α/β-hydrolase fold enzymes and a so-called "cap-domain", which is located on top of the core domain.

All epoxide hydrolases known to date require neither any prosthetic group nor a metal ion, and the mechanism by which these enzymes operate was long debated. Formerly, it was assumed that a direct nucleophilic opening of the oxirane ring by a histidine-activated water molecule would be the key step[71]. However, convincing

Figure 11.2-4. X-Ray structure of *Agrobacterium radiobacter* epoxide hydrolase (PDB-1EHY). The catalytic residues (Asp107 and His275) are located on top of the core-domain: at some distance Asp246 is shown, which is presumably involved in proton transfer. The α-helices at top left constitute the "cap-domain", which is covering the active site.

evidence was later provided which showed that the reaction occurs via a covalent glycol-monoester-enzyme intermediate[72, 73] (Fig. 11.2-5). For the *Agrobacterium* enzyme, the proposed active-site residues (Asp107 and His275) are located in the predominantly hydrophobic internal cavity between the core- and cap-domains[64, 70]. Furthermore, a tunnel filled with water molecules has been located, which leads to the back of the active site cavity. It is perfectly suited to deliver the catalytic water molecule within hydrogen-bonding distance to His275. Since the water is positioned at the back, the epoxide probably enters the active site from the front. In addition, Asp246 has been proposed as the third member of the catalytic triad (not shown in Fig. 11.2-5), since replacement of Asp107, His275 and Asp246 resulted in a dramatic loss of activity[64].

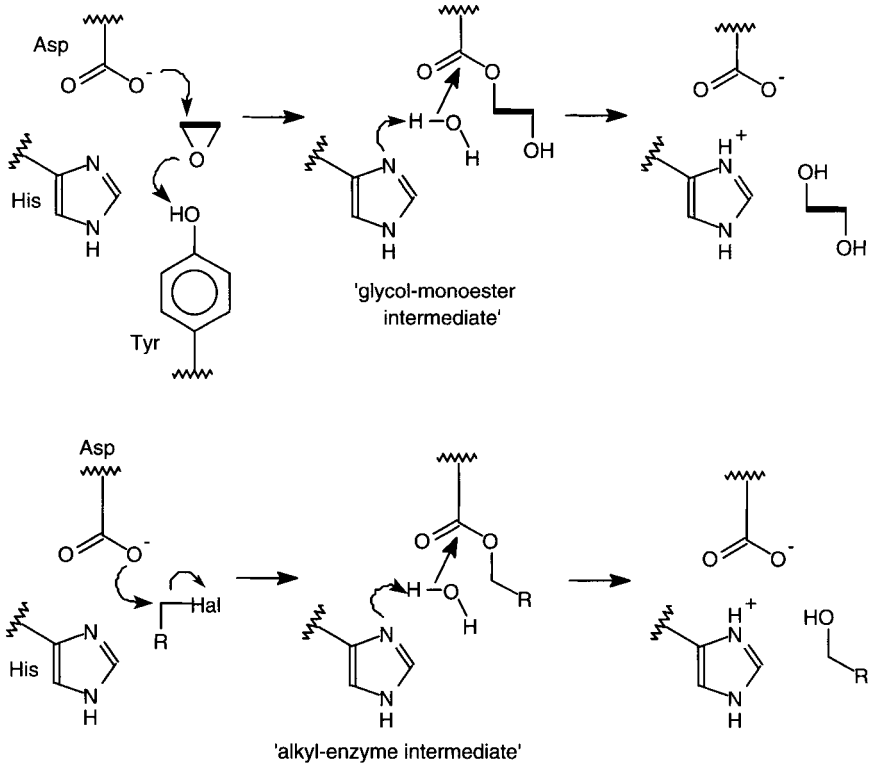

Figure 11.2-5. Schematic representation of the mechanism of epoxide hydrolase and of haloalkane dehalogenase.

Structure and mechanism show striking similarities to that of haloalkane dehalogenase from *Xanthobacter autotrophicus* (whose structure and mechanism has been substantiated by X-ray crystallography)[74, 78]. Both enzymes have an Asp-His-Asp catalytic triad which superimpose very well, their side chains point in a similar relative direction and they form analogous hydrogen bonds. In haloalkane dehalogenase, it has been shown that a halide is displaced from the substrate by an aspartate residue via a nucleophilic attack, thus leading to an "alkyl-enzyme intermediate" which is further hydrolyzed in a second step[75, 76]. Similar mechanisms have been proposed for other epoxide hydrolases belonging to the α/β-hydrolase fold family[38, 72, 77].

A consequence of the above-mentioned mechanism is a *trans*-specific opening of the epoxide with one oxygen from water being incorporated into the product diol[60]. For instance, (±)-*trans*-epoxysuccinate was converted into *meso*- (not D/L-) tartrate by an epoxide hydrolase isolated from *Pseudomonas putida*[60]. In a complementary fashion, *cis-meso*-epoxysuccinate gave D- and L-tartrate (with a *Rhodococcus* sp.) albeit in low optical purity[79]. The fact that only one O-atom originates from water was proven by ^{18}O-labelling experiments using bacterial, fungal[80] and mammalian epoxide hydrolases[81]. Although two cases for reactions proceeding via a formal *cis*-

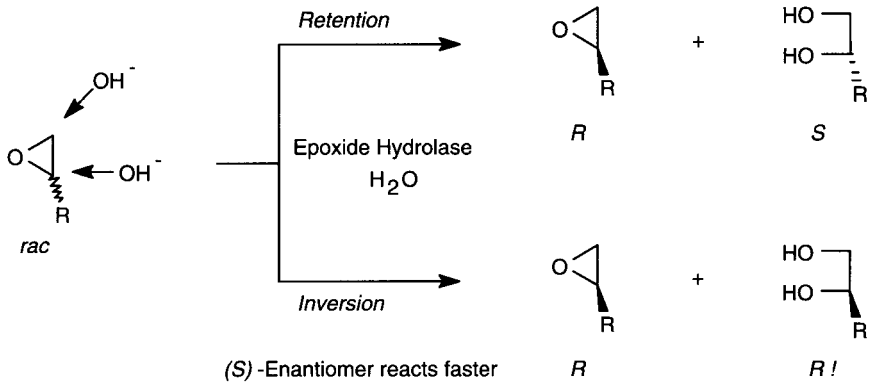

Figure 11.2-6. Microbial hydrolysis of epoxides proceeding with retention or inversion of configuration.

hydration process have been reported in the mid-1970s[82, 83], they seem to be rare exceptions and – given the present knowledge of the enzyme mechanism – attempts to explain this phenomenon remain rather speculative[83]. It is interesting to note that several β-glycosidases act via formation of a covalent glycosyl-enzyme intermediate by retaining the configuration at the anomeric centre[84]. This suggests that these enzymes may also be mechanistically related to epoxide hydrolases.

The above-mentioned facts have important consequences for the stereochemical outcome of the kinetic resolution of asymmetrically substituted epoxides. In the majority of enzymatic transformations following a kinetic resolution pattern (e.g. by ester hydrolysis and synthesis using lipases, esterases and proteases) the absolute configuration at the stereogenic centre(s) always remains the same throughout the reaction, since it is not directly involved in the reaction. In contrast, the enzymatic hydrolysis of epoxides may take place via attack on either carbon of the oxirane ring (Fig. 11.2-6) and it is the structure of the substrate and of the enzyme which determine the regioselectivity of the process[81, 85–89]. As a consequence, the absolute configuration of *both the product and the substrate* from a kinetic resolution of a racemic epoxide has to be determined in order to elucidate the stereochemical pathway. To facilitate the determination of this regioselectivity, a mathematical approach has been suggested, which only necessitates the study of the biohydrolysis of the racemic mixture[85].

11.2.1.3
Screening for Microbial Epoxide Hydrolases

In spite of the considerable value of epoxide hydrolases for fine chemical synthesis, it was only recently that a detailed search for epoxide hydrolases from microbial sources was undertaken by the groups of Furstoss[85, 90] and Faber[23, 79, 91], bearing in mind that the use of microbial enzymes allows an (almost) unlimited supply of biocatalyst. The screening was based along the following considerations: on the one hand, the catabolism of alkenes often implies the hydrolysis of an epoxide inter-

mediate and, on the other hand, detoxification of the highly reactive epoxy-intermediates is achieved via hydrolysis. As a consequence, it was anticipated that bacteria and fungi which were known to be able to epoxidize alkenes in an efficient manner should also possess a matching epoxide hydrolase activity. This proved to be true for the fungi *Aspergillus niger* and *Beauveria bassiana*, which were able to achieve the enantioselective hydrolysis of different types of epoxides derived from geraniol, limonene[90] or substituted styrene derivatives[92, 93]. In an extensive follow-up study, seven additional fungal strains (from a total of 42) were selected for exhibiting promising epoxide hydrolase activity[94]. The guidelines mentioned above were also successfully applied to the screening of bacteria, and strains were selected after a careful literature search based on the capability for alkene-epoxidation. Following the work described above, the occurrence of epoxide hydrolases in yeasts has been investigated[95, 96]. From a screening of 187 different yeast strains belonging to 25 different genera, 8 strains (*Trichosporon*, *Rhodotorula* and *Rhodosporidium* sp.) were identified by using 1,2-epoxyoctane as substrate[97]. The membrane-associated epoxide hydrolases from these yeasts show good enantioselectivities and high initial rates, especially for monosubstituted aliphatic epoxides[98].

It is noteworthy that, in contrast to mammalian systems, the majority of bacterial and fungal strains exhibited sufficient activity even when the cells were grown on a non-optimized standard medium. Since enzyme induction is still a largely empirical task, cells are usually grown on standard media in the absence of inducers. Furthermore, all attempts to induce epoxide hydrolase activity in *Pseudomonas aeruginosa* NCIMB 9571 and *Pseudomonas oleovorans* ATCC 29347 by growing the cells on an alkane (decane) or alkene (decene) as the sole carbon source failed[4]. Epoxide hydrolases from *Corynebacterium*[65] and *Rhodococcus* DCL4[69] seem to be exceptional with respect to their inducibility.

11.2.2
Microbial Hydrolysis of Epoxides

11.2.2.1
Fungal Enzymes

One of the first observations on microbial epoxide hydrolysis on a preparative scale was reported from the terpene field: thus, racemic geraniol *N*-phenylcarbamate was efficiently hydrolyzed by the fungus *Aspergillus niger*, yielding 42% of the remaining (6*S*)-epoxide in 94% *ee*. Interestingly, from the preparative point of view, this could easily be conducted on 5 g of substrate using a 7 L fermentor[90].

Similar results were obtained with styrene oxide, which was again very efficiently hydrolyzed by *A. niger*, thus affording the (*S*)-epoxide in 99% *ee* within a few hours[85]. In contrast, the fungus *Beauveria bassiana* (formerly *B. sulfurescens*) showed opposite enantioselectivity, leading to the (*R*)-epoxide in 99% *ee* (Fig. 11.2-7). In addition, interesting information concerning the mechanism implied in these transformations[80] and the scope of the substrates admitted could be established. Thus, it was shown that cyclic styrene analogs like *para*-substituted styrene oxide

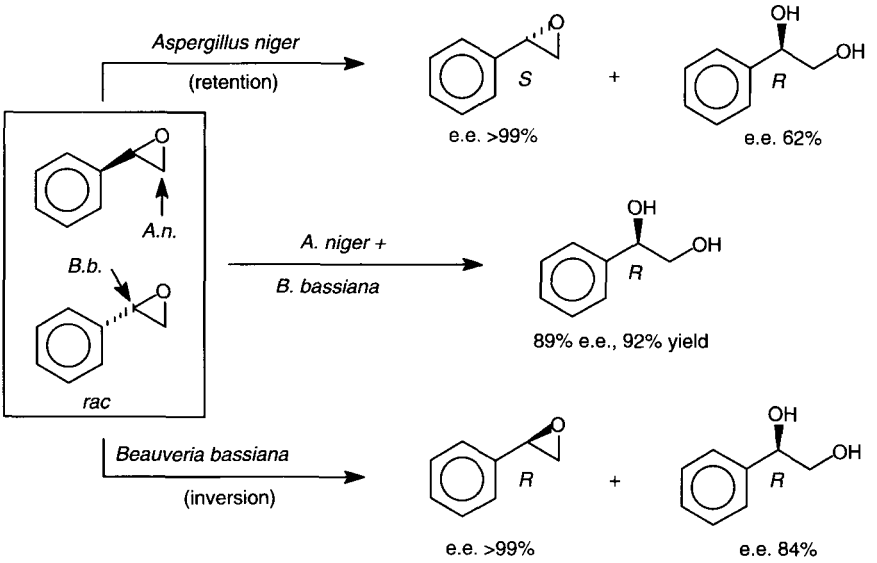

Figure 11.2-7. Resolution and deracemization of styrene oxide by fungal cells.

derivatives[99] or β-substituted analogs[92] were accepted by one – or both – of these fungi. During a subsequent study, seven additional fungi were tested on more than ten styrene oxide derivatives bearing various substituents[100]. It was shown that an increase of the size of the substituent resulted in a selectivity-enhancement, e.g., from $E = 3$ for styrene oxide to $E = 39$ for *para*-nitrostyrene oxide. However, a methyl substituent at Cα did not improve the enantioselectivity of the reaction.

Racemic epoxyindene was rapidly hydrolyzed when submitted to a culture of *B. sulfurescens*, leading to a 20% yield of recovered enantiomerically pure (*ee* >98%) (1*R*,2*S*)-epoxide, and to a 48% yield of the corresponding (1*R*,2*R*)-*trans*-diol showing 69% ee[101]. The latter product is of considerable importance for the synthesis of the HIV protease inhibitor indinavir. This prompted Merck Co. to perform a more extensive study of this biotransformation[102, 103], during which 80 fungal strains were evaluated for their ability to enantioselectively hydrolyze racemic epoxyindene. In a similar fashion, epoxydihydronaphthalene was successfully hydrolyzed to the corresponding (1*R*,2*R*)-diol in excellent enantiomeric purity[101].

Many of these fungal epoxide hydrolases were found to be soluble enzymes, which could be obtained as crude cell-free extracts and which could be stored at +4 °C without significant loss of activity. In this way, easy-to-use water-soluble catalysts were developed, which circumvented the problems often encountered when working with whole-cell mycelia[104, 105].

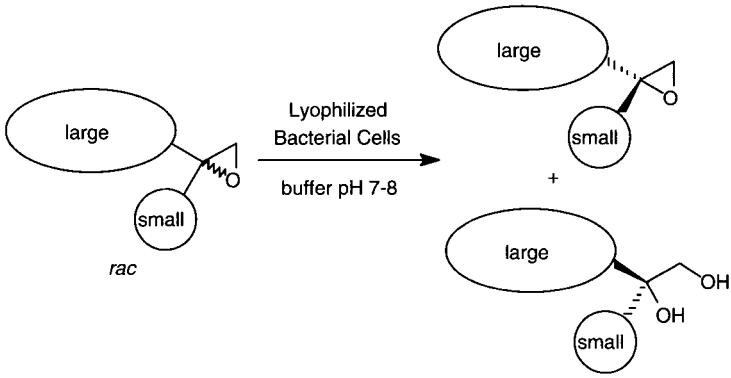

Figure 11.2-8. Resolution of 2,2-disubstituted oxiranes by bacterial cells.

11.2.2.2
Bacterial Enzymes

The use of bacterial cells for preparative biotransformations is particularly attractive for the following reasons: (i) they do not tend to form dense mycelia, which may impede agitation of large-scale reactions when whole-cell (fungal) systems are employed, and (ii) cloning of bacterial enzymes is generally less problematic.

However, disappointingly low selectivities were observed with monosubstituted aliphatic epoxides such as 1-epoxyoctane (E <5) or benzyl glycidyl ether (E <2)[60, 83]. On the other hand, the sterically more demanding 2,2-disubstituted oxiranes turned out to be much better substrates (Fig. 11.2-8, Table 11.2-1). Especially the substrates bearing a straight alkyl chain were transformed with virtually absolute selectivity, and functional groups such as a C=C-double bond[106] or a terminal bromo-group[107] were well tolerated. As a consequence of the exquisite enantioselectivity, the reactions ceased and did not proceed beyond a conversion of 50%.

Interestingly, the enantiopreference was found to depend on the substrate structure, but not on the strain used[108]. When the epoxide bears a synthetically useful phenyl moiety (mimicking a masked carboxyl function) at the ω-position of the alkyl chain, the selectivity was slightly reduced but still in a useful range (Table 11.2-1, last entry, E = 123)[107, 109]. Unexpectedly, when the carbon chain was extended by an

Table 11.2-1. Selectivities in the resolution of 2,2-disubstituted oxiranes by bacterial cells (see Fig. 11.2-8).

Small substituent	Large substituent	Biocatalyst	Selectivity (E)
CH_3	n-C_5H_{11}	*Nocardia* sp. EH1	> 200
CH_3	n-C_4H_9	*Nocardia* sp. TB1	> 200
CH_3	$(CH_2)_3CH = CH_2$	*Nocardia* sp. EH1	> 200
CH_3	n-C_7H_{15}	*Rhodococcus equi* IFO 3730	> 200
CH_3	n-C_9H_{19}	*Mycobacterium paraffinicum* NCIMB 10420	> 200
CH_3	$(CH_2)_4$-Br	*Nocardia* sp. H8	> 200
CH_3	CH_2Ph	*Nocardia* sp. EH1	123

Table 11.2-2. Selectivities in the resolution of 2,2-disubstituted oxiranes by whole cells of *Rhodococcus* sp. NCIMB 11216 (see Fig. 11.2-8).

Small substituent	Large substituent	Selectivity (*E*)
H	C_6H_{13}	2.8[a]
CH_3	C_5H_{11}	105
C_2H_5	C_5H_{11}	7
CH_3	C_7H_{15}	125
CH_3	C_9H_{19}	> 200
CH_3	CH_2-Ph	111
CH_3	$(CH_2)_2$-Ph	9.5

a With opposite absolute configuration.

additional CH_2-unit the selectivity declined (Table 11.2-2, last entry, E = 9.5). From an extensive study on *Rhodococcus* sp. NCIMB 11216 it was concluded that the enantioselectivity largely depends on the relative difference in size of the two alkyl substituent groups (Table 11.2-2). Increasing this size difference resulted in enhanced selectivities. The fact that the substrates bearing a phenyl group behave differently might be attributed to electronic effects, such as π-π-stacking. All of these biohydrolyses can be performed on a multigram-scale.

In contrast to the rather flexible bacterial epoxide hydrolases mentioned above, limonene-1,2-oxide hydrolase from *Rhodococcus erythropolis* DCL14, an enzyme involved in the limonene degradation pathway, has a rather narrow substrate specificity. Of the compounds tested, only the natural substrate limonene-1,2-oxide and several highly substituted (alicyclic) epoxides were substrates for the enzyme. The enantioselectivities were usually low, except for the natural substrate[110]. Styrene oxide, various derivatives thereof and phenyl glycidyl ether were obtained in high *ee* and reasonable yield using a recombinant epoxide hydrolase from *Agrobacterium radiobacter* AD1[111]. Interestingly, the Tyr152Phe and Tyr215Phe mutants showed a considerable increase in stereoselectivity. For example, the *E* value for *para*-chlorostyrene oxide increased fourfold from $E = 32$ for the wild-type enzyme to $E > 130$ for the Tyr215Phe mutant enzyme[78].

11.2.2.3
Yeast Enzymes

Yeasts are generally very sturdy microorganisms which are easy to cultivate on a large scale. These features make them interesting for preparative use[95]. Enantioselective epoxide hydrolysis by (red) yeasts has been studied only recently; the first report demonstrated the epoxide hydrolase activity of *Rhodotorula glutinis*[51] on several aryl, alicyclic and aliphatic epoxides. In follow-up studies, additional yeast strains exhibiting good activities and sufficient enantioselectivities were found, and the application of these biocatalysts has great potential[97, 98].

Given the data available to date, it seems to be a general phenomenon that epoxide hydrolases from fungi and from bacteria generally possess an opposite enantiopreference. Whereas epoxide hydrolases from fungi of matching opposite enantio-

preference are not known, an extensive screening showed that bacteria seem to be more flexible in this respect[112]. This allows one to control the stereochemical outcome by a simple choice of the appropriate microorganism.

11.2.3
Substrate Specificity and Selectivity

11.2.3.1
Asymmetrization of *meso*-Epoxides

The asymmetrization of a *meso*-epoxide via regioselective attack at one of the (enantiomeric) stereocenters of the oxirane would be an elegant application of epoxide hydrolases since it leads to a single *trans*-diol in 100% theoretical yield. Such asymmetrization reactions have been demonstrated with epoxide hydrolases from mammalian origin, which afforded the enantiomerically enriched corresponding diol[113, 114]. Unfortunately, few such reactions have been reported with microbial enzymes. For instance, cyclohexene oxide was hydrolyzed using *Corynesporium cassiicola* cells yielding *trans*-cyclohexane-1,2-diol, albeit with disappointingly low *ee* (27%)[115]. It was only after further metabolism involving an oxidation-reduction sequence by dehydrogenases present in the cells that the reaction product was transformed into optically pure (*S,S*)-cyclohexane-1,2-diol. In a related experiment, asymmetric hydrolysis of *cis*-epoxysuccinate using a crude enzyme preparation derived from *Rhodococcus* sp. led to D- and L-tartaric acid in almost racemic form[79]. Similar discouraging results were obtained using baker's yeast[116].

Recently, encouraging progress was made in the hydrolysis of cyclopentene oxide and cyclohexene oxide using the yeast *Rhodotorula glutinis*[95]. The corresponding (*R,R*)-*trans*-diols were obtained in over 90% optical and chemical yields. However, asymmetric hydrolysis of *meso*-epoxides by bacterial and fungal epoxide hydrolases is still impeded by insufficient selectivities.

11.2.3.2
Resolution of Racemic Epoxides

Monosubstituted Epoxides. Monosubstituted oxiranes [Fig. 11.2-9(a), Table 11.2-3] represent highly flexible and rather "slim" molecules, which make chiral recognition a difficult task. As a consequence, the majority of attempts using epoxide hydrolases from bacterial and fungal origin to achieve highly selective transformations failed[118] with one exception[117]. Most interestingly, the only selective enzymes were found among red yeasts, such as *Rhodotorula araucarae* CBS 6031[97], *Rhodo-*

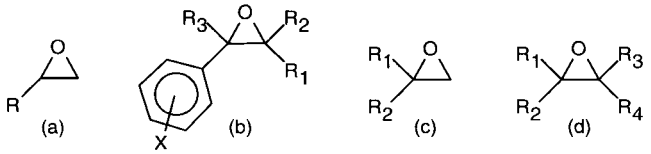

Figure 11.2-9. Substrate types (for selectivities see Tables 11.2-3-11.2-7).

Table 11.2-3. Enzymatic hydrolysis of monosubstituted epoxides, see Fig. 9(a).

R	Selectivity[a]	Enantiopreference	Enzyme Source[b]	Reference
n-C_3H_7, n-C_4H_9, n-C_5H_{11}, n-C_6H_{13}, n-C_8H_{17}, n-$C_{10}H_{21}$	±	*R*	BEH	117
CH_2Cl, $C(CH_3)_2O(CO)C(CH_3)_3$, CH_2OCH_2Ph	–	n.d.	BEH	8
t-C_4H_9, n-C_6H_{13}	–	*R*	BEH	23
n-C_6H_{13}	–	*R*	FEH	50
CH_3, C_2H_5	+	*R*	YEH	51
CH_2OH, CH_2Cl, CH_2OCH_2Ph	– to ±	*S*	YEH	51
n-C_6H_{13}	++	*R*	YEH	97
C_2H_5, n-C_3H_7, n-C_4H_9, n-C_5H_{11}, n-C_6H_{13}	++	*R*	YEH	96

a Selectivity denoted as (-) = low (E < 4), (±) = moderate (E = 4–12), (+) = good (E = 13–50), (++) = excellent (E > 50).
b BEH = bacterial epoxide hydrolase; FEH = fungal epoxide hydrolase; YEH = yeast epoxide hydrolase.
n.d. = not determined.

sporidium toluloides CBS 0349[97], *Trichosporon* sp. UOFS Y-01118[97] and *Rhodotorula glutinis* CIMW147[51]. These yeasts' epoxide hydrolase seem to have a preference for monosubstituted oxiranes with a chain length of approximately six carbon atoms or more (*E* up to 200). Furthermore, olefinic side chains are sometimes hydrolyzed selectively (*E* up to 100) as well[98]. Based on the rules of the kinetic resolution of a racemate, diols of high *ee* could only be obtained at low conversions[95]. With few exceptions, the enantiopreference for the (*R*)-configurated oxirane was predominant regardless of the enzyme source[118].

Styrene Oxide-Type Epoxides. Styrene-oxide-type oxiranes [Fig. 11.2-9(b), Table 11.2-4] have to be regarded as a special group of substrates, as they possess a benzylic carbon atom. This facilitates the formation of a carbocation which is stabilized by the adjacent aromatic moiety. As a consequence, nucleophilic attack at the benzylic position is electronically favored. On the other hand, the benzylic position is sterically more demanding, which favors the non-benzylic position. As a consequence, either oxirane carbon atom is easily attacked in this class of substrates and mixed regiochemical pathways are common. Since this results in reactions occurring with *inversion* and *retention* of configuration, *E*-values reported on these type of oxiranes have to be regarded with great caution whenever the regioselectivity has not clearly been elucidated. In order to achieve optimal enantioselectivities, the biocatalysts of choice for styrene oxide-type oxiranes are derived from red yeasts such as *Rhodutorula glutinis* CIMW 147[51, 95] and particularly from fungal epoxide hydrolases, e.g. *Aspergillus niger* LCP 521 and *Beauveria bassiana* ATCC 7159[118, 100]. The first entry in Table 11.2-4 is given solely for reason of comparison, since mammalian hepatic epoxide hydrolase was used. This enzyme source is not applicable to preparative-scale reactions.

Interestingly, the bacterial epoxide hydrolase from *Agrobacterium radiobacter* AD1 seems to hydrolyze *para*-substituted styrene oxides with opposite enantiopreference when compared to EHs from fungi or yeast[118]. Although initial selectivities were

Table 11.2-4. Enzymatic hydrolysis of styrene oxide-type epoxides, see Fig. 9(b).

R_1	R_2	R_3	X	Selectivity[a]	Enantio-preference	Enzyme source[b]	Reference
CH_3, C_2H_5, *n*-C_3H_7, *n*-C_4H_9, *n*-C_6H_{13}	H	H	H	++	1*S*[c]	mEH[d]	127
H	H	H	*p*-CH_3, *o*-Cl, *p*-Cl	±	*R*	BEH	111
H	H	CH_3	H	±	*R*	BEH	111
H	H	H	*p*-F, *p*-Cl, *p*-Br, *p*-CH_3	+	*S*	YEH	93
H	H	H	*p*-CH_3	+	*S*	YEH	93
H	H	H	*o*-CH_3, *o*-Hal	–	n.d.	YEH	93
H	H	H	H	±	*R*	YEH	51
H	CH_3	H	H	++	2*S*	YEH	51
H	H	H	H	++	*S*	FEH	92
H	H	CH_3	H	–	n.d.	FEH	92
indene oxide, dihydronaphthalene oxide				+	2*S*	FEH	92
H	CH_3	H	H	++	2*R*	FEH	92

a Selectivity denoted as (-) = low ($E < 4$), (±) = moderate ($E = 4–12$), (+) = good ($E = 13–50$), (++) = excellent ($E > 50$).
b BEH = bacterial epoxide hydrolase; FEH = fungal epoxide hydrolase; mEH = microsomal epoxide hydrolase from liver tissue; YEH = yeast epoxide hydrolase. n.d. = not determined.
c Enantioconvergent process (i.e. a single stereoisomeric diol was formed as the sole product).
d Performed on a microgram-scale only.

rather low[111], the *E*-values could be significantly increased by using specific mutants of the *Agrobacterium* enzyme[78].

Disubstituted Epoxides. Among the sterically more demanding substrates, 2,2-disubstituted epoxides were hydrolyzed with virtually absolutly enantioselectivities (E >200) using enzymes from bacterial sources [Fig. 11.2-9(c), Table 11.2-5]. In particular, *Actinomycetes* such as *Rhodococcus* and (closely related) *Nocardia* sp. are the biocatalysts of choice for this class of oxiranes[118]. Epoxide hydrolases from *Chryseomonas luteola*[117] and several fungi[50, 94] were less useful. Also for yeasts a 2-alkyl substituent resulted in a dramatic decrease in enantioselectivity[98]. In several cases, the regioselectivity of the reaction has been determined to be absolute. Attack occurs exclusively at the less hindered unsubstituted oxirane C-atom with complete retention at the stereogenic center. Most bacterial epoxide hydrolases showed a preference for the (*S*)-enantiomer. Only recently, it was shown that several methylotrophic bacterial strains exist, which show an opposite preference (i.e. for the (*R*)-epoxy enantiomer), albeit in moderate selectivities[112].

On the contrary, mixed regioselectivities were common when 2,3-disubstituted oxiranes were hydrolyzed and ring opening ocurred at both positions of the oxirane ring at various ratios (Table 11.2-6)[118]. This is understandable, bearing in mind that the steric requirements are similar at both positions. As a consequence, *E*-values are not applicable to the description of stereoselectivities. Again, *Actinomycetes* were found to be the catalysts of choice for this group of substrates[117]. Most remarkably, in selected studies, it was proven that the hydrolysis proceeded in an enantio-

Table 11.2-5. Enzymatic hydrolysis of 2,2-disubstituted epoxides, see Fig. 9(c).

R_1	R_2	Selectivity[a]	Enantio-preference	Enzyme Source[b]	Reference
CH_3	n-C_6H_{13}	–	n.d.	BEH	117
CH_3	n-C_5H_{11}	±	R or S[c]	FEH	50
C_2H_5	n-C_5H_{11}	±	S	BEH	8
CH_3	$(CH_2)_2Ph$	±	S	BEH	8
CH_3	CH_2Ph	+	S	BEH	8
CH_3	n-C_4H_9, n-C_5H_{11}, n-C_7H_{15}, n-C_9H_{19}	++	S	BEH	8
CH_3	$(CH_2)_4Br$	++	S	BEH	8
CH_3	$(CH_2)_3CH = CH_2$	++	S	BEH	8

a Selectivity denoted as (-) = low (E < 4), (±) = moderate (E = 4–12), (+) = good (E = 13–50), (++) = excellent (E > 50).
b BEH = bacterial epoxide hydrolase; FEH = fungal epoxide hydrolase; YEH = yeast epoxide hydrolase.
c Depending on the strain, the enantiopreference varied. n.d. = not determined.

Table 11.2-6. Enzymatic hydrolysis of 2,3-disubstituted epoxides, see Fig. 9(d).

R_1	R_2	R_3	R_4	Selectivity[a]	Enantio-preference	Enzyme Source[b]	Reference
H	n-C_4H_9, n-C_8H_{17}	H	n-C_8H_{17}, n-$C_{10}H_{21}$	++	2S	mEH	137
H	n-C_4H_9	H	$(CH_2)_{10}OH$	++	2S	mEH	137
H	n-C_8H_{17}	H	$(CH_2)_7CO_2H$	++	2S	mEH	137
H	CH_3, C_2H_5	H	n-C_4H_9, n-C_5H_{11}	++	2S	mEH[c]	138
H	$(CH_2)_2OH$	H	n-C_5H_{11}	++	2S	mEH[c]	138
H	$(CH_2)_2OCH_3$	H	n-C_5H_{11}	+	2S	mEH[c]	138
H	CH_3	H	n-C_5H_{11}	±	2S	FEH	50
CH_3	H	H	n-C_5H_{11}	±	2R/2S[d]	FEH	50
H	CH_3	CH_3	H	++	S	YEH	51
H	CH_3	H	CH_3	++	S	YEH	51
H	C_2H_5	H	n-C_4H_9	±	2S	BEH	8
C_2H_5	H	H	n-C_3H_7	±	1S	BEH	8
H	CH_3	H	n-C_4H_9	++	2S	BEH[c]	89
CH_3	H	H	n-C_4H_9, n-C_5H_{11}, n-C_6H_{13}	++	1S	BEH	8

a Selectivity denoted as (-) = low (E < 4), (±) = moderate (E = 4–12), (+) = good (E = 13–50), (++) = excellent (E > 50).
b BEH = bacterial epoxide hydrolase; FEH = fungal epoxide hydrolase; mEH = microsomal epoxide hydrolase from liver tissue; YEH = yeast epoxide hydrolase.
c Enantioconvergent process (i.e. a single stereoisomeric diol was formed as the sole product).
d Depending on the strain, the enantiopreference varied.

convergent fashion, and only one stereoisomeric diol was formed as the sole product. In contrast, fungi seem less appropriate for the hydrolysis of 2,3-disubstituted oxiranes[94], whereas *Rhodotorula glutinis* was more effective on the *cis*-configurated analogs of this substrate class[95]. Interestingly, in contrast to the bacteria, this yeast seems to operate via a classic kinetic resolution rather than an enantioconvergent pathway. In this way, the simple choice of the appropriate microorganism gives access to either the optically pure epoxide (yeast) or the optically pure diol (bacteria).

Table 11.2-7. Enzymatic hydrolysis of trisubstituted epoxides, see Fig. 9(d).

R_1	R_2	R_3	R_4	Selectivity[a]	Enantio-preference[b]	Enzyme Source[c]	Reference
H	$(CH_2)_2C(OAc)(CH_3)C$ $H{=}CH_2$	CH_3	CH_3	+	1*S*	BEH	119
H	Ph	CH_3	CH_3	–	–	FEH	92
	1,2-limonene oxide			++	*S*	YEH	51
	1-methylcyclohexene oxide			++	2*S*	BEH	121

a Selectivity denoted as (–) = low (E < 4), (±) = moderate (E = 4–12), (+) = good (E = 13–50), (++) = excellent (E > 50).
b Configuration of preferentially attacked oxirane carbon atom.
c BEH = bacterial epoxide hydrolase; FEH = fungal epoxide hydrolase; YEH = yeast epoxide hydrolase.

Trisubstituted Epoxides. To date, only a limited set of data are available on the enzymatic hydrolysis of trisubstituted epoxides (Table 11.2-7). Regardless of their steric bulkiness, however, they seem to be accepted by epoxide hydrolases from bacterial[110, 119], fungal[92, 94] and yeast[95] sources, as long as the access to one side of the substrate is not too severely restricted (e.g. a 2,2-dimethyl-3-alkyloxirane). Further data are required to depict a general selectivity pattern within this group of substrates.

11.2.3.3
Deracemization Methods

In contrast to the asymmetrization of *meso*-epoxides, which would lead to the highly desirable formation of a single stereoisomeric vicinal diol in 100% theoretical yield, the kinetic resolution of racemic epoxides by fungal and bacterial cells has proven to be highly selective (see above). However, this latter technique forms both the unreacted epoxide and the corresponding vicinal diol in equal amounts. This so-called classic kinetic resolution pattern of the biohydrolysis is often regarded as a major drawback, since the theoretical chemical yield can never exceed 50% based on the racemic starting material. As a consequence, methods that offer a solution to this intrinsic problem are highly advantageous[120]. Several procedures which overcome this drawback have been reported in the last few years.

For instance, based on the finding that styrene oxide could be resolved by whole cells of *Aspergillus niger* and *Beauveria bassiana* via two different pathways showing matching enantio- and regioselectivities a deracemization was developed: thus, combination of both biocatalysts in a single reactor led to (*R*)-phenylethane-1,2-diol as the sole product in 98% *ee* and 85% isolated yield[85] (Fig. 11.2-7).

Another strategy for the achievement of an enantioconvergent process was set up using the combination of bio- and chemo-catalysis[107, 109, 121, 122]. For instance, 2,2-disubstituted epoxides were selectively resolved by lyophilized whole cells of *Nocardia* sp. The biohydrolysis proceeds *via* attack at the less substituted C-atom with excellent regioselectivity thus leading to *retention* of configuration at the stereogenic center. On the other hand, acid-catalyzed hydrolysis of such epoxides usually proceeds at the more substituted oxirane carbon with *inversion*. Careful

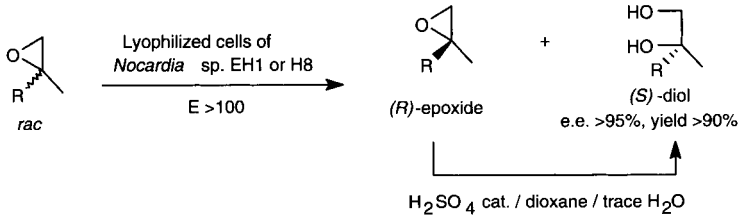

Figure 11.2-10. Resolution-inversion sequence for the deracemization of 2,2-disubstituted oxiranes involving the remaining epoxide.

combination of both catalytic steps (Fig. 11.2-10) in a resolution-inversion sequence yields the corresponding (*S*)-1,2-diols in virtually enantiopure form and in high yields (> 90%)[107, 109]. In a similar fashion, racemic *para*-nitrostyrene oxide was deracemized using a crude enzymatic extract from *Aspergillus niger* (Fig. 11.2-11). In this case a 4:1 water-DMSO solvent mixture was used, showing that this enzyme is operative in the presence of water miscible organic solvents. The resolution step was followed by the careful addition of acid, leading to (*R*)-*para*-nitrostyrene diol in good yield (94%) and *ee* (80%). Because of the reduced enantioselectivity and the fact that racemization occurred to a certain extent during the acidic hydrolysis, it was necessary to tune both catalytic steps very carefully. A mathematical method was therefore developed that made it possible to select the optimum conversion at which the acid hydrolysis step should be initiated[122, 123]. Careful mechanistic analysis of the acidic hydrolysis reaction, using different solvents and mineral acids, made it possible to select general conditions for the resolution-inversion procedure[107]. As a consequence, large scale deracemization became feasible[124].

The compatibility of microbial epoxide hydrolases with organic solvents deserves a special comment. It has been reported that in the majority of cases, the addition of water-miscible or -immiscible organic (co)solvents has negative effects on the activity. This is particularly true for bacterial enzymes, which showed total deactivation[124]. On the other hand, several epoxide hydrolases from yeasts and fungi seem

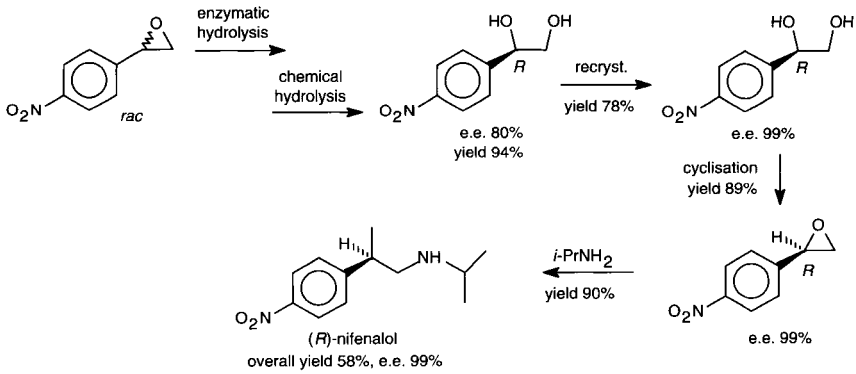

Figure 11.2-11. Deracemization of *para*-nitrostyrene oxide by a chemoenzymatic process. Application to the synthesis of (*R*)-Nifénalol®.

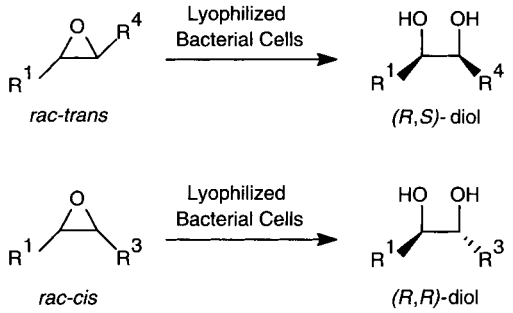

Figure 11.2-12. Resolution and deracemization of 2,3-disubstituted oxiranes by bacterial cells.

to be less sensitive and are able to tolerate water-miscible cosolvents, such as DMSO at a low level[125].

Cases for a non-classic deracemization of racemic epoxides using *one single biocatalyst* impose high requirements on matching regio- and enantioselectivities, and are therefore rare. For instance, the enantioconvergent hydrolysis of (±)-3,4-epoxytetrahydropyran[126] and several *cis*-β-alkyl substituted styrene oxides[127] by hepatic microsomal epoxide hydrolase has been reported on an analytical scale. Similarly, soybean epoxide hydrolase converted (±)-*cis*-9,10-epoxy-12(*Z*)-octadecenoic and (±)-*cis*-12,13-epoxy-9(*Z*)-octadecenoic acid into the corresponding (*R,R*)-dihydroxy acids as the sole products[128]. However, enantioconvergent hydrolysis on a synthetically useful scale was only reported recently. Thus, the fungus *Beauveria bassiana* transformed (±)-*cis*-β-methyl styrene oxide in an enantioconvergent manner to afford (1*R*,2*R*)-1-phenylpropane-1,2-diol in 85% yield and 98% *ee*[92]. In a related fashion, 2,3-disubstituted epoxides were hydrolyzed by using the *Nocardia* EH1 (Table 11.2-8)[89, 129]. Thus, the biohydrolysis of *cis*-2,3-epoxyheptane furnished (*R,R*)-*threo*-2,3-heptane diol in 79% isolated yield and 91% *ee* on a gram scale. In the latter study, the four stereochemical pathways and the enzyme mechanism were elucidated by $^{18}OH_2$-labeling experiments. The hydrolysis was shown to proceed by attack of a (formal) hydroxyl ion at the (*S*)-configurated oxirane carbon atom with concomitant *inversion* of configuration at *both enantiomers with opposite regioselectivity.* In addition, a mathematical model for the kinetics which allows the optimization of such enantioconvergent processes in preparative applications was developed.

Table 11.2-8. Selectivities in the deracemization of 2,3-disubstituted oxiranes by bacterial cells, see Fig. 9(d).

R_1	R_2	R_3	R_4	Biocatalyst	Configuration of diol	ee [%]
n-C_4H_9	H	H	CH_3	*Nocardia* EH1	2*R*, 3*S*	90
n-C_3H_7	H	H	C_2H_5	*Arthrobacter* sp. DSM 312	2*R*, 3*S*	63
n-C_5H_{11}	H	H	CH_3	*Rhodococcus* sp. NCIMB 11216	2*R*, 3*S*	77
n-C_6H_{13}	H	H	CH_3	*Rhodococcus* sp. NCIMB 11216	2*R*, 3*S*	78
n-C_4H_9	H	CH_3	H	*Nocardia* EH1	2*R*, 3*R*	97
n-C_3H_7	H	C_2H_5	H	*Nocardia* TB1	2*R*, 3*R*	77

11.2.4
Use of Non-Natural Nucleophiles

In reactions catalyzed by hydrolytic enzymes of the serine-hydrolase type, which form covalent acyl-enzyme intermediates during the course of the reaction, it has been shown that the "natural" nucleophile (water) can be replaced with "foreign" nucleophiles[130] such as an alcohol, amine, hydroxylamine, hydrazine and even hydrogen peroxide. As a consequence, a wealth of synthetically useful reactions, which are usually performed in organic solvents at low water content, can be performed in a stereoselective manner. Although one requirement is fulfilled by epoxide hydrolases – i.e. a covalent enzyme-substrate intermediate is formed – the sensitivity of epoxide hydrolases to most of the water-miscible or -immiscible organic solvents[49, 124] poses a general problem in the use of non-natural nucleophiles in enzymatic epoxide hydrolysis. However, two types of transformations, i.e. the aminolysis and azidolysis of an epoxide have been reported for selected cases (Fig. 11.2-13).

When racemic aryl glycidyl ethers were subjected to aminolysis in aqueous buffer catalyzed by hepatic microsomal epoxide hydrolase from rat, the corresponding (*S*)-configurated amino-alcohols were obtained in 51–88% *ee*[131]. On the other hand, when azide was employed as nucleophile for the asymmetric opening of 2-methyl-1,2-epoxyheptane in the presence of an immobilized crude enzyme preparation derived from *Rhodococcus* sp., which contains an epoxide hydrolase activity, the reaction revealed a complex picture[132]. The (*S*)-epoxide from the racemate was hydrolyzed (as in the absence of azide), and the less readily accepted (*R*)-enantiomer was transformed into the corresponding azido-alcohol (*ee* >60%). Although at present only speculations can be made about the actual mechanism of both the aminolysis and azidolysis reaction, in both cases it was proven that the reaction was catalyzed by a protein and that no reaction was observed in the absence of biocatalyst

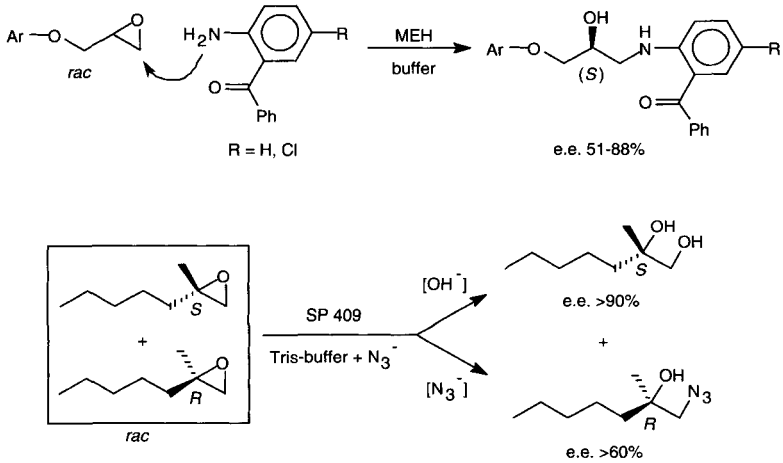

Figure 11.2-13. Enzyme-catalyzed aminolysis and azidolysis of epoxides.

or by using a heat-denatured preparation. However, a recent related report on the aminolysis of epoxides employing crude porcine pancreatic lipase[133] may likewise be explained by catalysis of a chiral protein surface rather than true lipase catalysis, since the latter enzyme – being a serine hydrolase – is irreversibly deactivated by epoxides. In view of these facts, it remains questionable whether the use of non-natural nucleophiles will be of general applicability with epoxide hydrolases.

11.2.5
Applications to Asymmetric Synthesis

Although the use of an epoxide hydrolase for the asymmetric hydrolysis was reported for industrial synthesis of L- and *meso*-tartaric acid as early as 1969[60], it was only recently that applications to asymmetric synthesis appeared in the literature. This fact can be attributed to the limited availability of these biocatalysts from sources such as mammals or plants. Since the production of large amounts of crude enzyme is now feasible, preparative-scale applications are getting within reach of the synthetic chemist. For instance, fermentation of *Nocardia* EH1 on a 70-L scale afforded >700 g of lyophilized cells[62].

One of the first applications of the microbial hydrolysis of epoxides for the synthesis of a bioactive compound is based on the the resolution of a 2,3-disubstituted epoxy-fatty acid having a *cis* configuration (Fig. 11.2-14). Thus, by using an enzyme preparation from *Pseudomonas* sp., the (9*R*,10*S*)-enantiomer was hydrolyzed in a *trans*-specific fashion (i.e. via inversion of configuration at C-10) yielding the (9*R*,10*R*)-*threo*-diol. The remaining (9*S*,10*R*)-epoxide was converted into (+)-disparlure, the sex pheromone of the gypsy moth in >95% *ee*[134].

Another illustration of the use of such a biocatalytic approach was the synthesis of either enantiomer of α-bisabolol. One of the stereoisomers is of industrial value for the cosmetic industry. This approach was based on the diastereoselective hydrolysis of a mixture of oxirane-diastereoisomers obtained from (*R*)- or (*S*)-limonene (Fig. 11.2-15)[90]. Thus, starting from (*S*)-limonene, the biohydrolysis of the mixture of (4*S*,8*RS*)-epoxides led to unreacted (4*S*,8*S*)-epoxide and (4*S*,8*R*)-diol. The former

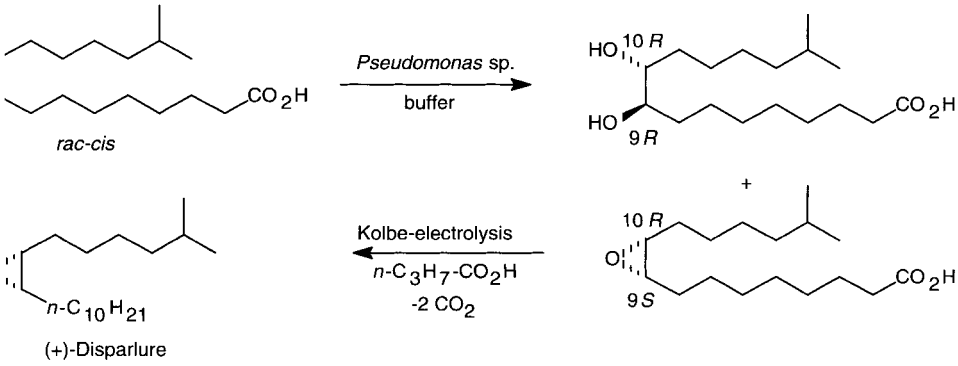

Figure 11.2-14. Resolution of a *cis*-2,3-disubstituted epoxide and synthesis of disparlure.

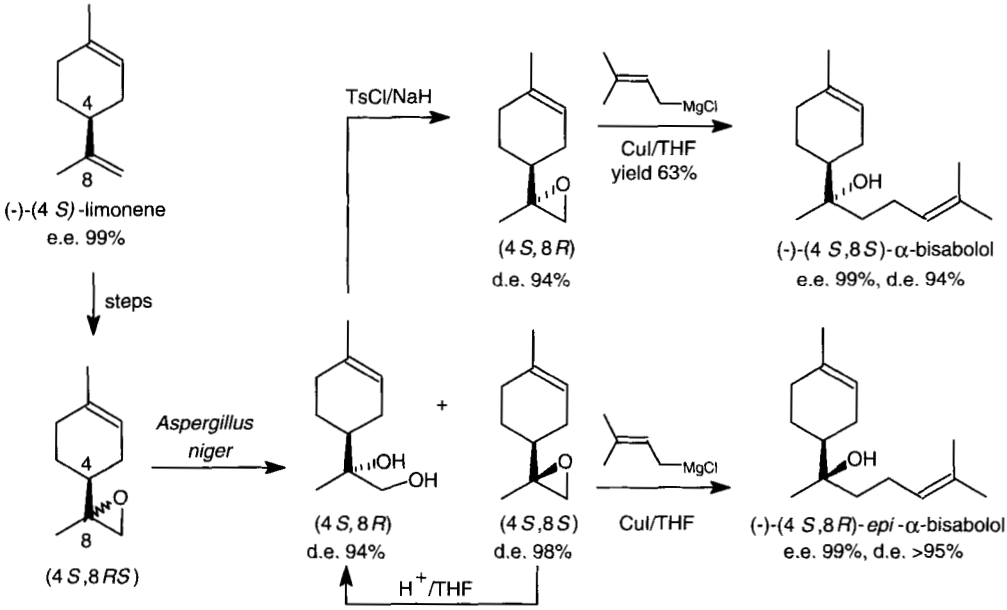

Figure 11.2-15. Chemoenzymatic synthesis of α-bisabolol using fungal epoxide hydrolase.

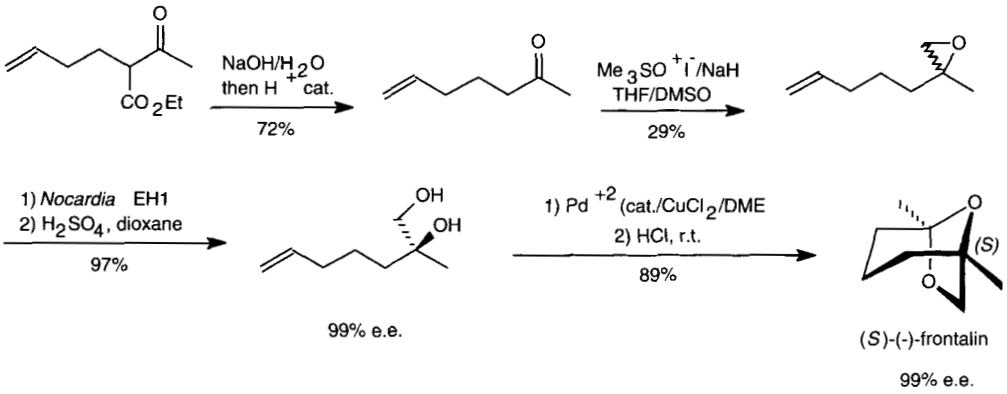

Figure 11.2-16. Chemoenzymatic synthesis of (*S*)-(-)-frontalin using bacterial epoxide hydrolase.

showed a high diastereomeric purity (*de* >95 %) and was chemically transformed into (4*S*,8*S*)-α-bisabolol. The formed diol (*de* >94 %) could be cyclized back to the corresponding (4*S*,8*R*)-epoxide, thus affording access to another stereoisomer of α-bisabolol. In addition, the two remaining stereoisomers of bisabolol could be prepared in a similar manner starting from (*R*)-limonene.

Based on the deracemization of (±)-3-methyl-2-(4-pentenyl)-oxirane (Fig. 11.2-16) using *Nocardia* EH1 and sulfuric acid in dioxane containing a trace amount of water (see above), (*S*)-2-methyl-hept-6-ene-1,2-diol was obtained in 97 % yield and 99 % *ee*[109]. This intermediate was successfully applied in a short synthesis of (*S*)-

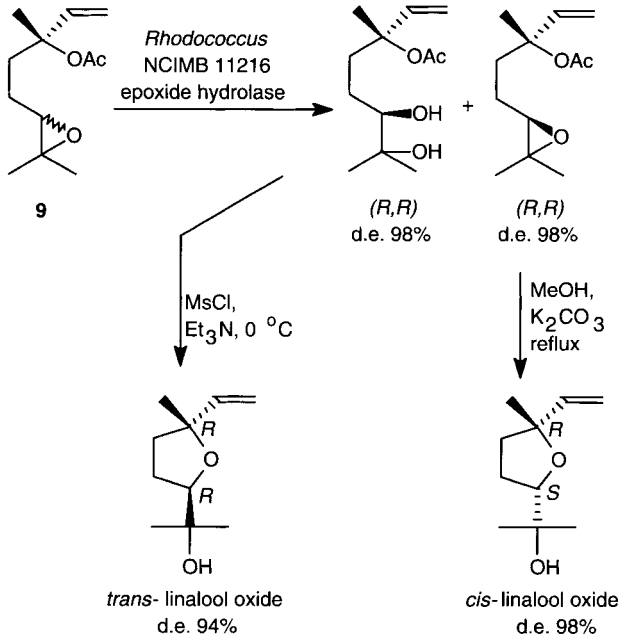

Figure 11.2-17. Synthesis of *cis*- and *trans*-linalool oxide.

(–)-frontalin, a central aggregation pheromone of pine beetles of the *Dendroctonus* family[106].

Enantiopure *cis*- and *trans*-linalool oxides are found in several plants and fruits and constitute the main aroma components of oolong and black tea. These compounds were prepared from 2,3-epoxylinalyl acetate (Fig. 11.2-17)[119]. The key step consists of a separation of the diastereomeric mixture of the starting epoxide by employing an epoxide hydrolase preparation derived from *Rhodococcus* sp. NCIMB 11216, which furnished the product diol and remaining epoxide in excellent diastereomeric excess (*de* >98%). Further follow-up chemistry gave both linalool oxide isomers on a preparative scale in excellent diastereomeric and enantiomeric purities.

Both enantiomers of the biologically active Bower's compound, a potent analog of insect juvenile hormone[135], were prepared using *Aspergillus* sp. cells in 96% *ee* (Fig. 11.2-18). Subsequent biological tests showed that the (6*R*)-antipode was about ten times more active than the (6*S*)-counterpart against the yellow meal worm *Tenebrio molitor*.

Aspergillus niger was the biocatalyst of choice for the biohydrolysis of *para*-nitrostyrene oxide (see above). A selective kinetic resolution using a crude enzyme extract of this biocatalyst, followed by careful acidification of the cooled crude reaction mixture, afforded the corresponding (*R*)-diol in high chemical yield (94%) and good *ee* (80%). This key intermediate could then be transformed via a four-step sequence into enantiopure (*R*)-nifenalol, a molecule with β-blocker activity, which was obtained in 58% overall yield (Fig. 11.2-11)[89, 122].

The natural (*R*)-(–)-isomer of mevalonolactone, a key intermediate in a broad spectrum of cellular biological processes and their regulation, was synthesized via

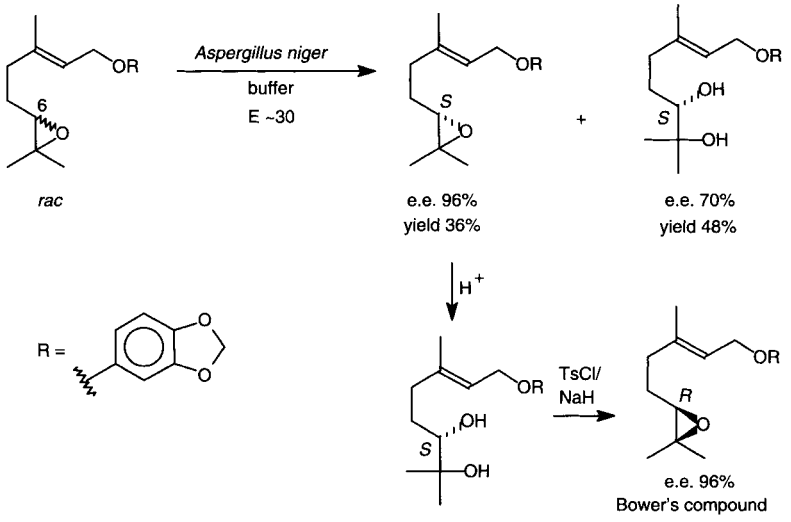

Figure 11.2-18. Synthesis of Bower's compound.

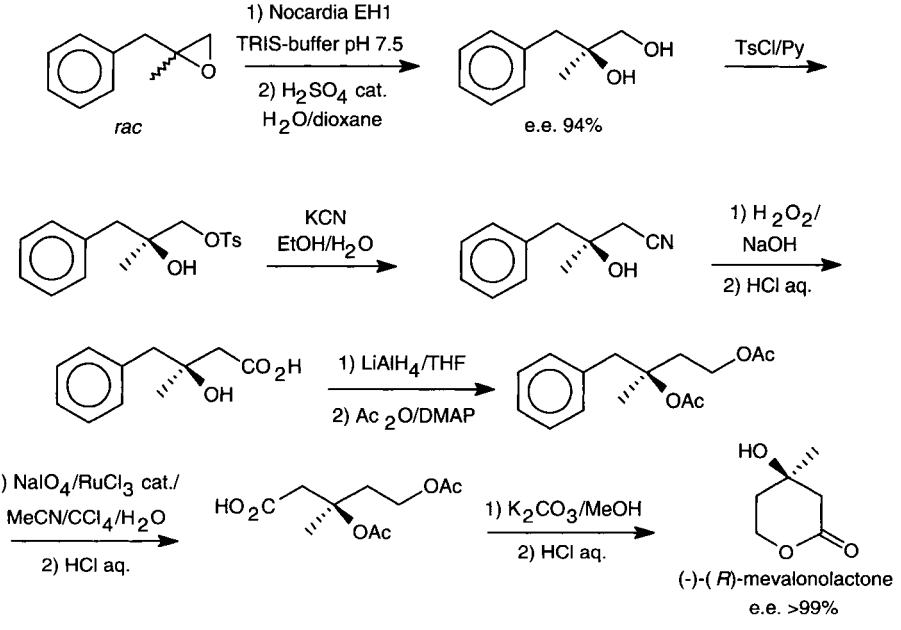

Figure 11.2-19. Synthesis of (*R*)-(-)-mevalonolactone.

eight steps in 55% overall yield and >99% *ee* (Fig. 11.2-19). In the key step, the aforementioned enantioconvergent chemoenzymatic deracemization route was applied. Thus, 2-methyl-2-benzyl-oxirane was deracemized on a 10 g scale using lyophilized cells of *Nocardia* EH1 and sulfuric acid. The product (*S*)-diol was isolated in 94% chemical yield and 94% optical purity[124]. During the scale-up of this

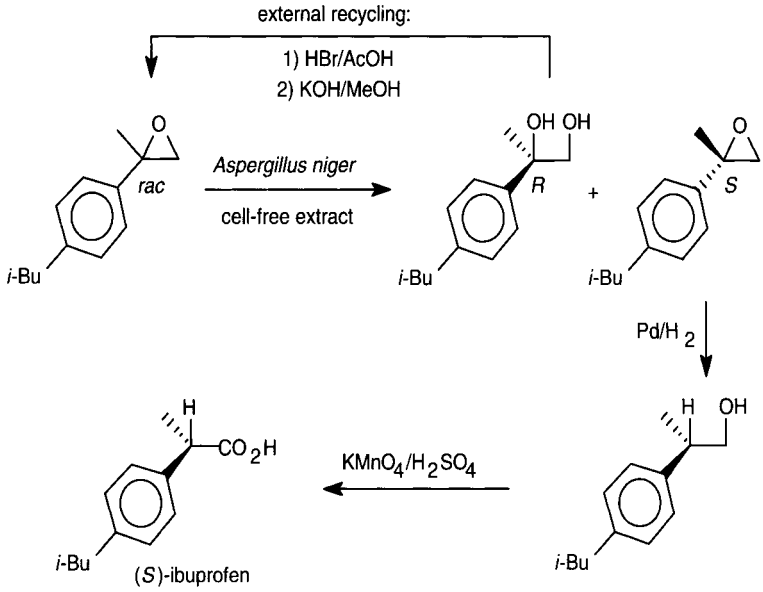

Figure 11.2-20. Chemoenzymatic synthesis of (S)-ibuprofen.

biotransformation it was observed that the increase of the substrate concentration led to a fourfold enhancement of the enantioselectivity as compared to analytical scale test reactions [124].

Finally, a chemoenzymatic enantioconvergent procedure led to (*S*)-ibuprofen in four steps and 47 % overall yield (Fig. 11.2-20). The latter compound is a widely used antiinflammatory drug and pain remedy and is one of the top ten drugs sold worldwide [100]. In the key step, the conditions for the enantioconvergent hydrolysis of *para-iso*-butyl-α-methylstyrene oxide was optimized (elevated substrate concentration at +4 °C) to afford the non-reacted epoxide in >95 % *ee* [136]. After separation from the epoxide, the formed diol (70 % *ee*) was recycled via a two-step sequence via the corresponding bromohydrin, which was cyclized back to give (±)-epoxide. The latter material was subjected to repeated biocatalytic resolution in order to improve the economy of the process.

11.2.6
Summary and Outlook

Over the past few years, an impressive array of epoxide hydrolases has been identified from microbial sources. Due to the fact that they can be easily employed as whole-cell preparations or crude cell-free extracts in sufficient amounts by fermentation, they are just being recognized as highly versatile biocatalysts for the preparation of enantiopure epoxides and vicinal diols. The future will certainly bring an increasing number of useful applications of these systems to the asymmetric synthesis of chiral bioactive compounds. As for all enzymes, the enantioselectivity of

microbial epoxide hydrolysis depends on the substrate structure and the type of enzyme involved. The data available to date indicate that the enantioselectivites of enzymes from certain microbial sources can be roughly correlated to the substitutional pattern of various types of substrates: red yeast give best selectivities with monosubstituted oxiranes, fungal cells are suited for styrene oxide-type substrates and bacterial enzymes are the catalysts of choice for more highly substituted 2,2- and 2,3-disubstituted epoxides. Since the first three-dimensional X-ray structure of an epoxide hydrolase has recently been solved, more will follow, which will improve the predictability of stereoselectivities. Given the data presented above, possible industrial applications of microbial epoxide hydrolases can be anticipated in the near future.

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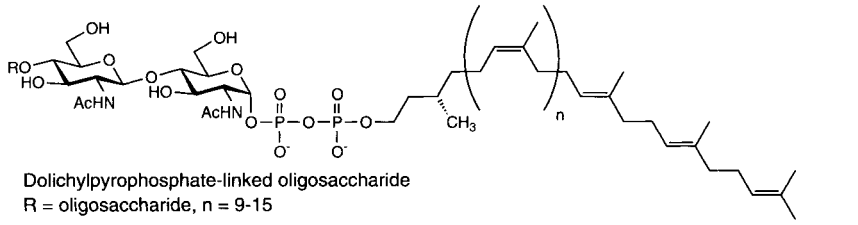
11.3
Hydrolysis and Formation of Glycosidic Bonds

Chi-Huey Wong

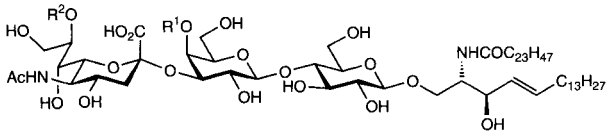
1.3.1
Introduction

Carbohydrates are found in nature as components of a broad range of molecular structures[1–50]. Attached to cell surface glycoproteins and glycolipids, they play vital roles in cellular communication processes[1–23], function as points of attachment for proteins such as antibodies, and serve as receptor sites for bacteria and viral particles[12, 13, 52]. For example, the sialyl-Lewis X tetrasaccharide mediates the adhesion of neutrophils to the endothelial layer, an initial event in the inflammatory response[44, 45, 53–56]. Glycoprotein glycans can modulate protein folding and are involved in the sorting and trafficking of proteins to appropriate cellular sites[1, 21, 40, 41, 57, 58].

Nature employs two groups of enzymes in the biosynthesis of oligosaccharides, namely those of the Leloir[59–62] and non-Leloir pathways. Leloir enzymes are responsible for the synthesis of most glycoproteins and other glycoconjugates in mammalian systems. Glycoprotein glycans are typically classified as either *N*-linked or *O*-linked, based on the linkage between the carbohydrate and the protein. *N*-linked glycans are characterized by a β-glycosidic linkage between GlcNAc and an asparagine δ-amide nitrogen. The majority of *O*-linked glycans contain an α-glycosidic linkage between GalNAc and a serine or threonine hydroxyl group. The addition of oligosaccharide chains to glycoproteins occurs post- or co-translationally in the endoplasmic reticulum and the Golgi apparatus[60]. *N*-linked oligosaccharides all contain the same basic core structure composed of GlcNAc and mannose residues. *N*-linked glycan biosynthesis involves the initial construction of a dolichyl pyrophosphoryl oligosaccharide intermediate in the endoplasmic reticulum catalyzed by GlcNAc-transferases and mannosyltransferases. This structure is then glucosylated, presumably as a signal for transfer of the oligosaccharide to the polypeptide. The entire oligosaccharide moiety is then transferred *en bloc* to an Asn residue of the growing peptide chain catalyzed by oligosaccharyltransferase[60, 63, 64]. The Asn is typically part of the amino acid sequence Asn-X-Ser(Thr), where X≠Pro or Asp[30, 60, 65–67]. Before transport into the Golgi apparatus, trimming of the glycan by glucosidases I and II and a mannosidase reveals a core pentasaccharide (peptide-Asn-$(GlcNAc)_2$-$(Man)_3$). This structure is further processed by mannosidases and glycosyltransferases in the Golgi apparatus, resulting in either a high-mannose, complex, or hybrid type oligosaccharide. Sequential addition of monosaccharides then provides the fully-elaborated oligosaccharide chain. In contrast, the more structurally diverse *O*-linked glycans are assembled within the Golgi apparatus by glycosyltransferases[21, 60]. In the most common route, GalNAc is initially appended to serine or threonine catalyzed by a UDP-GalNAc:polypeptide GalNAc-transferase. Monosaccharides are then added individually to the growing oligosaccharide chain by glycosyltransferases.



Dolichylpyrophosphate-linked oligosaccharide
R = oligosaccharide, n = 9-15

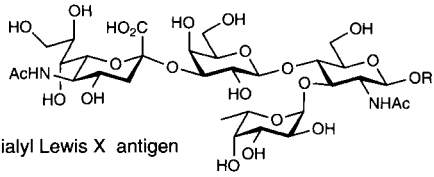


Ganglioside GM_1: R^1 = Gal 1,3GalNAc -, R^2 = H

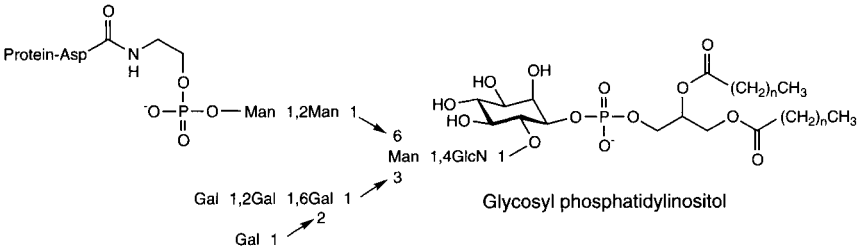
Ganglioside GM_2: R^1 = GalNAc -, R^2 = H

Ganglioside GM_3: R^1 = H, R^2 = H

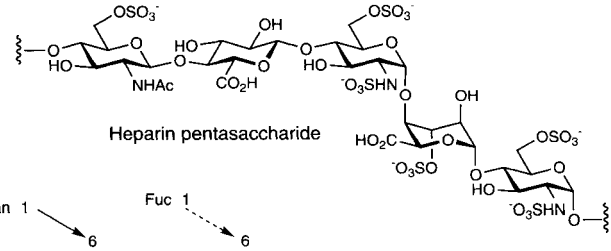
Ganglioside GD_3: R^1 = H, R^2 = NeuAc -



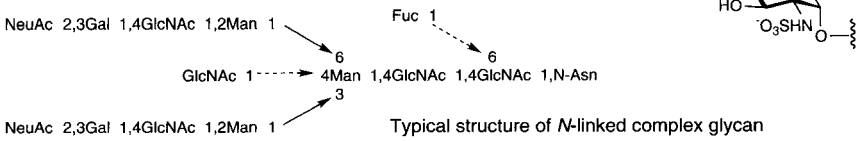
Sialyl Lewis X antigen



Glycosyl phosphatidylinositol



Heparin pentasaccharide



Typical structure of *N*-linked complex glycan

Figure 11.3-1.

All mammalian cells, with the exception of erythrocytes, contain the necessary elements for glycosylation. In certain secretory cells, however, the preponderance of glycosyltransferases is greater [68]. The structures of some typical naturally-occurring glycoproteins, glycolipids, and oligosaccharides are illustrated in Fig. 11.3-1.

The major classes of cell-surface glycolipids include the glycosphingolipids (GSLs) and glycoglycerolipids. Gangliosides [69], or sialic acid-containing glycosphingolipids, are especially abundant on neural cell surfaces [70]. These compounds play a role in the differentiation of cell types and in the regulation of cell growth. Additionally, sphingosine, the lipid component of GSLs, has been suggested to function as an intracellular second messenger [71].

The mammalian glycosyltransferases of the Leloir pathway utilize monosaccharides activated as glycosyl esters of nucleoside mono- or diphosphates as glycosyl donor substrates [60]. Primarily eight nucleotide sugars serve as glycosyl donors for the synthesis of most oligosaccharides: UDP-Glc, UDP-GlcNAc, UDP-Gal, UDP-GalNAc, GDP-Man, GDP-Fuc, UDP-GlcUA, and CMP-NeuAc (Fig. 11.3-1). Many other monosaccharides, such as the anionic or sulfated sugars of heparin and chondroitin sulfate, are also found in mammalian systems, but usually are the result of post-glycosyl transfer modifications [1, 27, 72a,b]. Non-Leloir glycosyltransferases typically employ glycosyl phosphates as activated donors. A diverse array of monosaccharides (e.g. xylose, arabinose, KDO) and oligosaccharides is also present in microorganisms, plants, and invertebrates [33, 62, 73–76]. The enzymes responsible for their biosynthesis, however, have not been extensively exploited for synthesis, though the same principles as in mammalian systems apply. Some sugar nucleotides used by enzymes of other pathways are also shown in Fig. 11.3-2.

Chemists have employed glycosyltransferases from the Leloir and non-Leloir pathways for the synthesis of oligosaccharides and glycoconjugates [77–83]. Glycosidases have also been exploited for synthesis [77–84]. The function of glycosidases *in vivo* is to cleave glycosidic bonds; however, under appropriate conditions, they can be useful synthetic catalysts. Each group of enzymes has certain advantages and disadvantages for synthesis. Glycosyltransferases are highly specific in the formation of glycosides, but the availability of many of the necessary enzymes is limited. Glycosidases have the advantage of wider availability and lower cost, but they are not as regio-specific or high-yielding in synthetic reactions. Therefore the chemist must choose the enzyme which is best suited for the application at hand. Other enzymatic methods used to synthesize glycoconjugates will also be discussed.

11.3.2
Glycosyltransferases of the Leloir Pathway

Glycosyltransferases are highly regiospecific and stereospecific with respect to the formation of new glycosidic linkages. Although also usually substrate-specific, minor chemical modifications are tolerated on both the donor and acceptor components. The preparative use of glycosyltransferases has been somewhat limited in the past because of a lack of enzyme availability. Additionally, because glycosyltransferases are membrane-bound enzymes, they are relatively unstable and can be

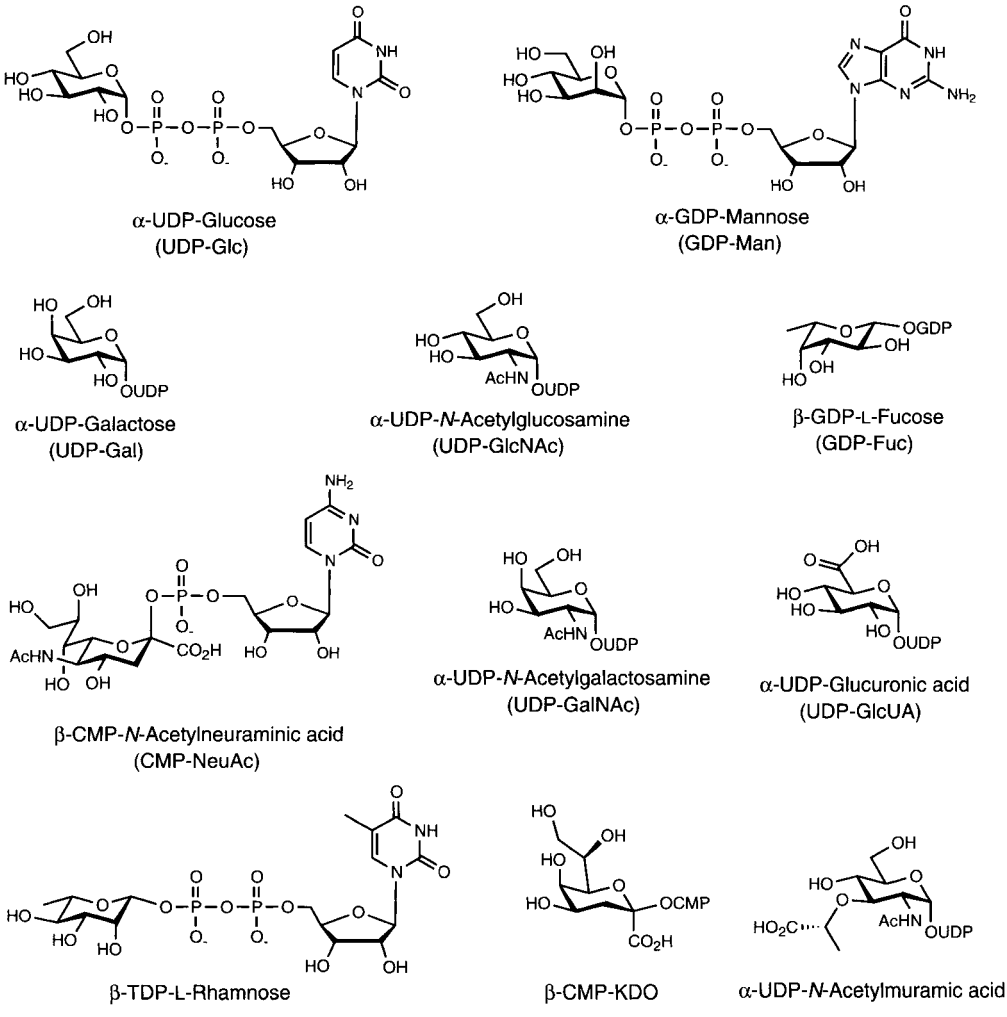


Figure 11.3-2.

difficult to handle in solution. However, the recent isolation of many of these enzymes, as well as advances in genetic engineering and recombinant techniques, are rapidly alleviating these drawbacks.

Glycosyltransferases utilize nucleotide sugars as activated glycosyl donors[60]. Most of these sugar nucleoside phosphates are biosynthesized *in vivo* from the corresponding monosaccharides (Fig. 11.3-3). The initial step is kinase-mediated phosphorylation to produce a glycosyl phosphate. This glycosyl phosphate then reacts with a nucleoside triphosphate (NTP), catalyzed by a nucleoside diphosphosugar pyrophosphorylase, to afford an activated nucleoside diphosphosugar [Eq. (1)]. Some sugar nucleoside phosphates, such as GDP-Fuc and UDP-GlcUA, are biosynthesized by further enzymatic modification of existing key sugar nucleotides.

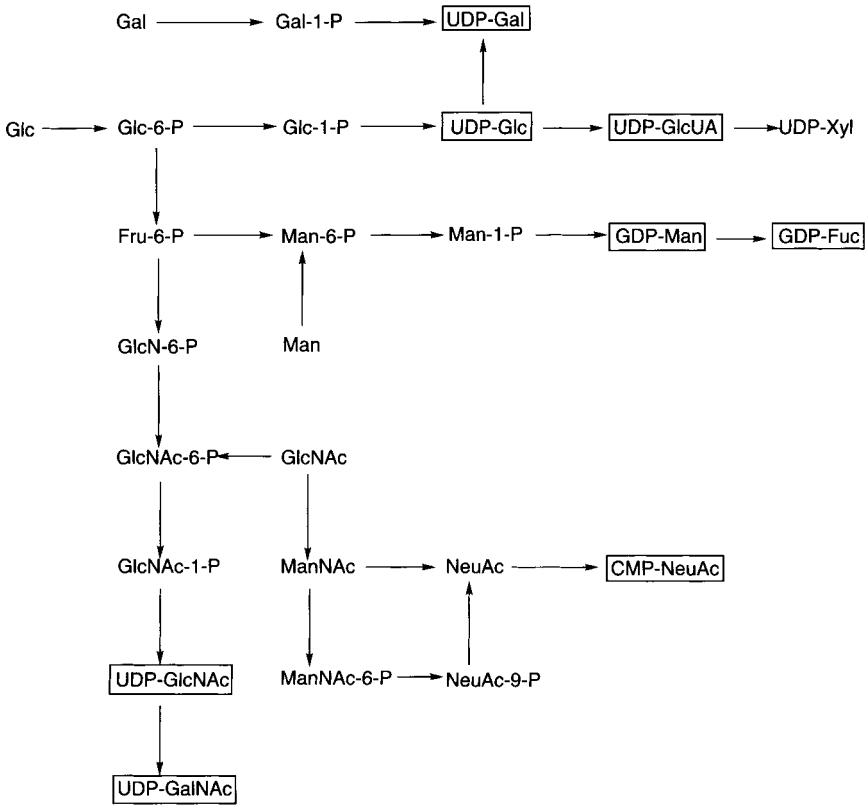


Figure 11.3-3.

In contrast, CMP-NeuAc is formed by the condensation of NeuAc with CTP [Eq. (2)]. Some of the enzymes involved in the biosynthesis of sugar nucleotides also accept unnatural sugars as substrates. In general, however, the rates are quite slow, thus limiting the usefulness of this approach.

$$\text{Sugar-1-P} + \text{NTP} \rightarrow \text{NDP-Sugar} + \text{PP}_i \quad (1)$$

$$\text{NeuAc} + \text{CTP} \rightarrow \text{CMPNeuAc} + \text{PP}_i \quad (2)$$

11.3.2.1

Synthesis of Sugar Nucleoside Phosphates

Chemical syntheses of some sugar nucleoside phosphates have been reported[85]. Most of these methods involve the reaction of an activated NMP[86–91] with a glycosyl phosphate to produce a sugar nucleoside diphosphate (Fig. 11.3-4). Of the commonly used activated NMP derivatives, phosphoramidates such as phosphorimidazolidates[92–94] and phosphoromorpholidates[86–91] are considered the most effective. A recent improvement in coupling methodology employing *1H*-tetrazole as catalyst has been reported[95]. These activated NMPs may also be used to prepare

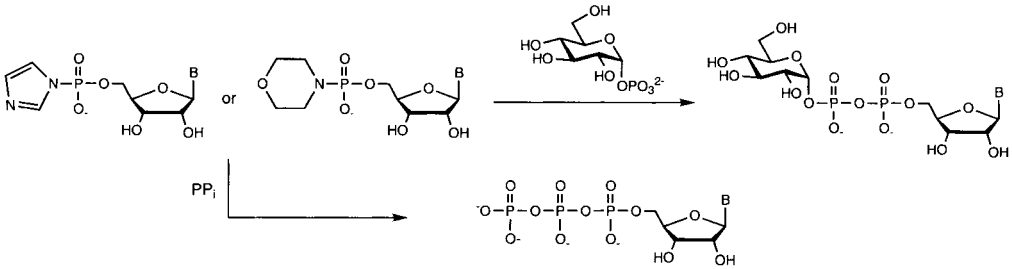
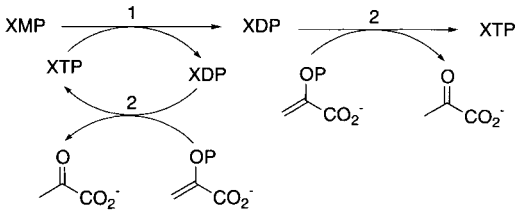


Figure 11.3-4.



1. Adenylate kinase (EC 2.7.4.3, X = A,C,U)

   Guanylate kinase (EC 2.7.4.8, X = G)

   Nucleoside monophosphate kinase (EC 2.7.4.4, X = U)

2. Pyruvate kinase (EC 2.7.1.40)

Figure 11.3-5.

NTPs by reaction with pyrophosphate (Fig. 11.3-5)[88]. A number of chemical methods are available for the synthesis of glycosyl phosphates. Reactions of phosphates with activated glycosyl donors[89, 96] or chemical phosphorylation of anomeric hydroxyl groups[89–92, 97] have proven to be convenient. Additionally, routes via glycosyl phosphites are useful[98]. Enzymatic procedures include employing glycogen phosphorylase[99] and sucrose phosphorylase[100] for the production of α-glucose-1-phosphate. Phosphoglucomutase can also be used to prepare glucose-1-phosphate from glucose-6-phosphate[101], the latter generated from glucose by hexokinase catalysis.

Preparative-scale synthesis of nucleoside triphosphates. Nucleoside triphosphates are utilized as substrates for the biosynthesis of sugar nucleoside phosphates. Practical-scale biosynthesis-based enzymatic preparation of NTPs for use in glycosylations is therefore required.

Most preparative-scale enzymatic syntheses of NTPs use commercially available NMPs as starting materials. Alternatively, NMPs can be obtained from yeast RNA digests at low cost[102], or can be easily prepared chemically[103]. In general, enzymatic methods involve the sequential use of two kinases to transform NMPs to NTPs, via the corresponding NDPs. Several kinases have been utilized to synthesize NTPs from the corresponding NDPs, each employing a different phosphoryl donor: pyruvate kinase (E.C. 2.7.1.40) uses phosphoenolpyruvate (PEP)[104, 105] as a phosphoryl donor, acetate kinase (E.C. 2.7.2.1) uses acetyl phosphate, and nucleoside

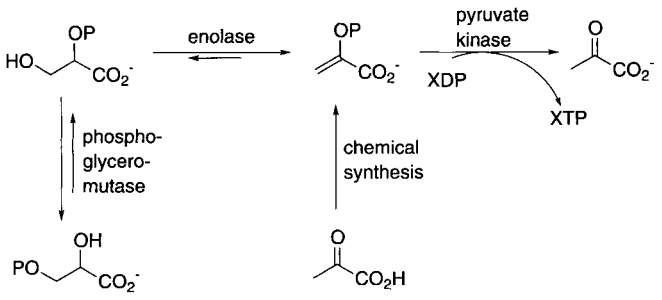


Figure 11.3-6.

diphosphate kinase (E. C. 2.7.4.6) uses ATP. Pyruvate kinase has generally been the enzyme of choice because it is less expensive than nucleoside diphosphate kinase[77, 94, 107], and because PEP is more stable and provides a more thermodynamically favorable driving force for phosphorylation than does acetyl phosphate (Fig. 11.3-5). A recently described polyphosphate kinase uses polyphosphate as donor, providing a potentially cheaper kinase route[106].

The preparation of NDPs from NMPs is more complicated, and requires different enzymes for each NMP. Adenylate kinase (E. C. 2.7.4.3) phosphorylates AMP[88] and CMP[108], and also slowly phosphorylates UMP. Guanylate kinase (E.C. 2.7.4.8) catalyzes the phosphorylation of GMP. Nucleoside monophosphate kinase (E. C. 2.7.4.4) uses ATP to phosphorylate AMP, CMP, GMP, and UMP; however, the enzyme is relatively expensive and unstable[94]. Both CMP and UMP kinases exist but are not commercially available. For those kinases requiring ATP as a phosphorylating agent, ATP is usually used in a catalytic amount and recycled from ADP using pyruvate kinase/PEP or acetate kinase/acetylphosphate[77, 109]. Phosphoenolpyruvate may be prepared chemically from pyruvate[104] or generated enzymatically from D-3-phosphoglyceric acid[105] (Fig. 11.3-6).

When chemical and enzymatic methods for NTP synthesis are compared[94], enzymatic techniques provide the most convenient route to CTP and GTP, whereas chemical deamination of CTP is the best method for preparing UTP[94]. ATP is relatively inexpensive from commercial sources, although it has been synthesized enzymatically from AMP on 50 mmol scale. Mixtures of NTPs can be prepared from RNA by sequential nuclease P_1, polynucleotide phosphorylase, and pyruvate kinase-catalyzed reactions[110]. This mixture can be selectively converted to a sugar nucleotide using a particular sugar nucleoside diphosphate pyrophosphorylase[110].

UDP-glucose (UDP-Glc) and UDP-galactose (UDP-Gal). UDP-glucose has been prepared from UTP and glucose-1-phosphate under catalysis by UDP-glucose pyrophosphorylase (Fig. 11.3-7)[94, 101, 113, 114]. UDP-Gal can be synthesized in an analogous fashion using UDP-Gal pyrophosphorylase[101], or from UDP-Glc by epimerization of C-4 with UDP-glucose epimerase[101] (Fig. 11.3-7). Though the epimerase equilibrium favors UDP-Glc, the reaction can be coupled to an *in situ* glycosylation with galactosyltransferase to shift toward UDP-Gal production. The latter process has been applied to large-scale synthesis of *N*-acetyllactosamine (LacNAc)[101]. UDP-Gal has been prepared from UMP and Gal using dried cells of

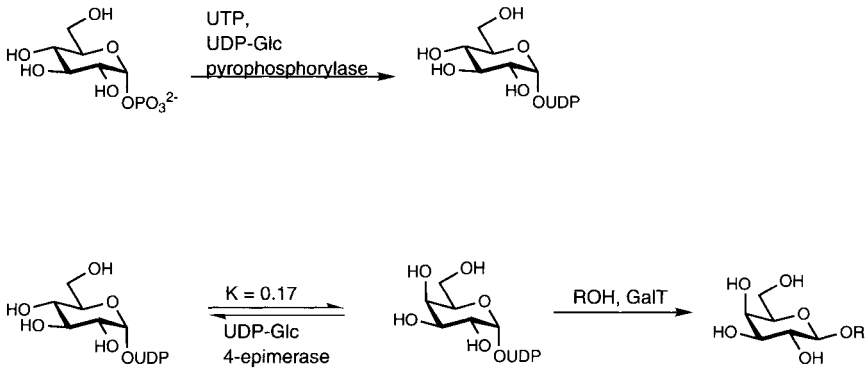


Figure 11.3-7.

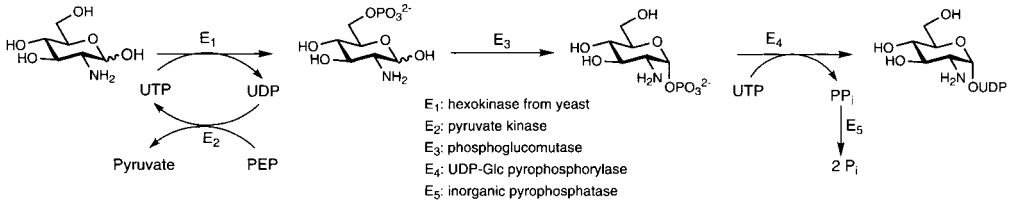


Figure 11.3-8.

Torulopsis candida[111]. In this system, Gal-1-phosphate and UTP were generated *in situ* as substrates for UDP-Gal pyrophosphorylase. Gram quantities of UDP-Gal, as well as the 2-fluoro-UDP-Gal derivative have been synthesized by an enzymatic method employing Gal-1-P uridyltransferase[116].

UDP-N-acetylglucosamine (UDP-GlcNAc). UDP-GlcNAc has been synthesized by reaction between GlcNAc-1-phosphate and UTP, catalyzed by UDP-GlcNAc pyrophosphorylase[111]. Although this enzyme is currently not commercially available, a whole-cell process using baker's yeast can be employed[111]. Another procedure exploits UDP-Glc pyrophosphorylase to catalyze a condensation between UTP and glucosamine-1-phosphate (GlcN-1-P) to afford UDP-glucosamine[112] (Fig. 11.3-8). The product UDP-GlcN can then be selectively *N*-acetylated to provide UDP-GlcNAc. GlcN-1-P has been synthesized from GlcN by phosphorylation of the 6-position with hexokinase to give GlcN-6-P, followed by a phosphoglucomutase-catalyzed isomerization to provide GlcN-1-P. UDP-GlcNAc also serves as an acceptor for β1,4-GalT to provide UDP-LacNAc[117].

UDP-N-acetylgalactosamine (UDP-GalNAc). The biosynthetic enzymes UDP-GalNAc pyrophosphorylase and UDP-GlcNAc 4-epimerase are not readily available for facile synthesis of UDP-GalNAc. An alternative synthetic procedure based on UMP exchange between UDP-Glc and GalN-1-P, catalyzed by commercially available UDP-Glc: galactosylphosphate uridyltransferase (E.C. 2.7.7.12) has been reported (Fig. 11.3-9)[62, 113]. Galactose-1-P is the natural substrate for the enzyme, but 2-deoxygalactose-1-P, 2-deoxyglucose-1-P, and galactosamine-1-P are also tolerated.

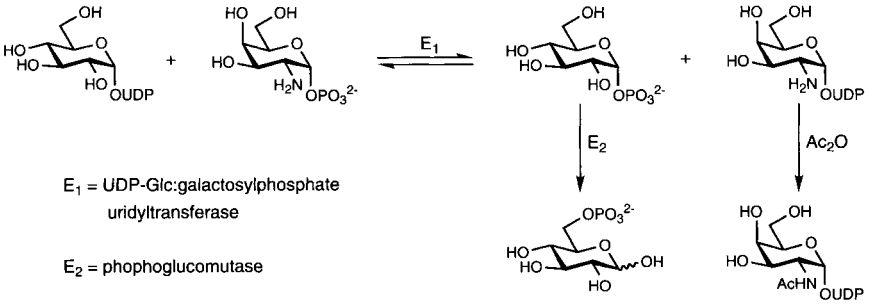


Figure 11.3-9.

As the equilibrium constant for the exchange reaction is close to unity, phosphoglucomutase was required to relieve product inhibition and shift the equilibrium. The UDP-GalN thus produced was then chemically acetylated to give UDP-GalNAc.

A modification of the latter procedure has been adapted to large-scale synthesis of UDP-GalNAc[118]. In this procedure, UDP-Glc was regenerated *in situ* from UTP and the product Glc-1-P under catalysis by UDP-Glc pyrophosphorylase. This also shifts the equilibrium toward the formation of UDP-GalN. Alternatively, UDP-GalNAc can be prepared from UMP and sucrose employing sucrose synthase[119]. Large-scale production of UDP-GalNAc in yeast has also been accomplished[120].

GDP-mannose (GDP-Man). GDP-mannose has been prepared from Glc and GMP using dried baker's yeast cells[111]. The procedure involves the biocatalytic conversion of glucose to Man-1-P and subsequently to GDP-Man using GDP-Man pyrophosphorylase. A cell-free extract from baker's yeast has also been used to synthesize GDP-Man from mannose[121]. A direct synthesis from chemically-prepared Man-1-P and GTP, catalyzed by GDP-Man pyrophosphorylase (E.C. 2.7.7.13) is useful for large scale producation (Fig. 11.3-10)[94]. Alternative strategies for continuous GDP-Man production[122], the synthesis of GDP-Man directly from mannose[123], and other routes have also been pursued[124].

GDP-fucose (GDP-Fuc). GDP-fucose is biosynthesized *in vivo* from GDP-Man by an NADPH-dependent oxidoreductase enzyme system. Such systems have also been utilized for *in vitro* syntheses of GDP-Fuc. For example, the synthesis of GDP-Fuc was accomplished using a crude enzyme preparation from *Agrobacterium radiobacter*[125]. NADPH was regenerated *in situ* from NADP using glucose-6-phosphate dehydrogenase and Glc-6-P[126]. Employing a similar procedure, GDP-Fuc has been

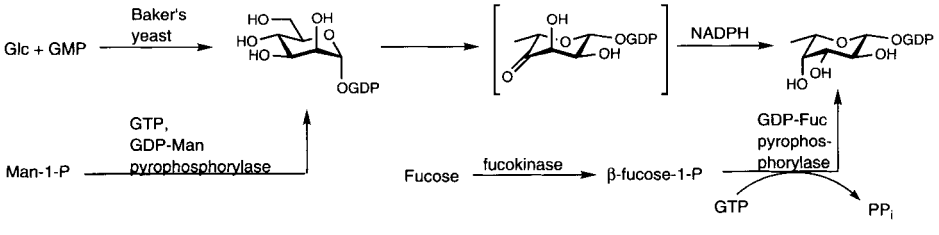


Figure 11.3-10.

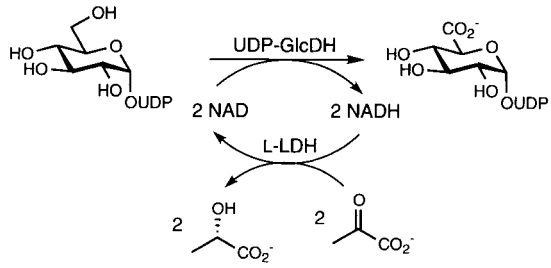


Figure 11.3-11.

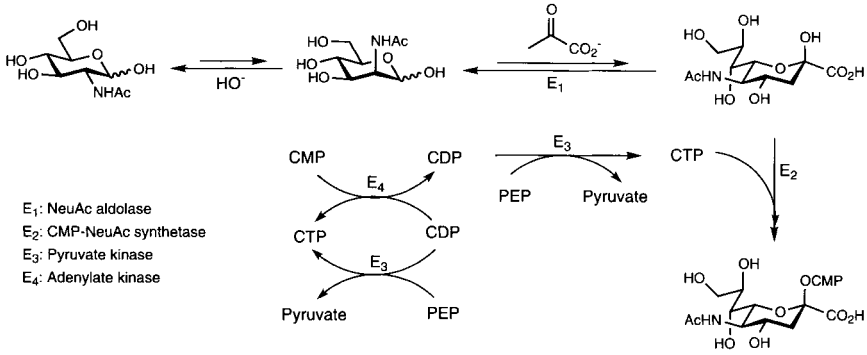


Figure 11.3-12.

generated *in situ* for use in a glycosylation reaction with α-1,3-fucosyltransferase[127]. Enzymes from a minor biosynthetic pathway which synthesize GDP-Fuc from L-fucose have also been exploited for synthesis[127]. Fucose was phosphorylated by fucokinase (E.C. 2.7.1.52) to produce Fuc-1-P, which subsequently underwent a GDP-fucose pyrophosphorylase-catalyzed reaction with GTP to provide GDP-Fuc. Several practical chemical syntheses of GDP-Fuc have also been reported[95, 128].

UDP-glucuronic acid (UDP-GlcUA). UDP-Glucuronic acid is biosynthesized by C-6 oxidation of UDP-Glc with UDP-Glc dehydrogenase, an NAD-dependent enzyme. Enzyme preparations from bovine liver have been employed for gram-scale syntheses of UDP-GlcUA (Fig. 11.3-11)[94, 129]. The NAD cofactor was regenerated with lactate dehydrogenase in the presence of pyruvate. Additionally, extracts from guinea pig liver have been used to generate UDP-GlcUA *in situ* for use in enzymatic glycosylations with glucuronyltransferases[130].

CMP-N-acetylneuraminic acid (CMP-NeuAc). CMP-*N*-acetylneuraminic acid has been prepared enzymatically on small scales (> 0.5 mmol) from CTP and NeuAc, under catalysis by CMP-NeuAc synthetase (EC 2.7.7.43)[131]. An improvement in this procedure, involving *in situ* production of CTP from CMP under adenylate kinase and pyruvate kinase catalysis, is suitable for multigram-scale synthesis[132]. Adenylate kinase catalyzes the equilibration of CTP and CMP to CDP, which is subsequently phosphorylated by pyruvate kinase to provide CTP. A one-pot synthesis of CMP-NeuAc based on this procedure involves the *in situ* synthesis of NeuAc from *N*-acetylmannosamine and pyruvate, catalyzed by sialic acid aldolase (Fig. 11.3-12)[108]. Chemical syntheses of CMP-NeuAc have also been reported[142].

The gene encoding *E. coli* CMP-NeuAc synthetase[133, 134] has been cloned into the Lambda ZAP vector and overexpressed in *E. coli* at a level 1000 times that of the wild type[307,308]. The enzyme from calf brain has also been cloned and overexpressed. CMP-NeuAc synthetase was shown to accept several NeuAc derivatives as substrates. For example, 9-deoxy-, 7,9-dideoxy-, and 4,7,9-trideoxy-NeuAc are all converted to the corresponding CMP-NeuAc derivatives[137]. On the other hand, the 4-oxo, 7-oxo, and 8-oxo NeuAc derivatives are not substrates for CMP-NeuAc synthetase[138]. However, the enzyme accepts a variety of modifications at the 9-position, and the hydroxyl group can be replaced with several different groups with little effect on the K_M value[139–141]. CMP-NeuAc can also be obtained on the large scale by fermentation[143] or by coupling of metabolically engineered bacterial cells[144].

11.3.2.2
Substrate Specificity and Synthetic Applications of Glycosyltransferases

For each sugar nucleotide glycosyl donor, many glycosyltransferases of varying substrate specificities exist. These enzymes are generally considered to be specific for a given glycosyl donor and acceptor, as well as for the stereochemistry and the linkage position of the newly formed glycoside bond. This specificity has led to the "one enzyme-one linkage" concept[28, 142, 161]. In other words, the specificity of the glycosyltransferases ensures fidelity in oligosaccharide sequences *in vivo* without the use of a template scheme. Though systematic investigations of the *in vitro* substrate specificity of most glycosyltransferases have not been carried out, some deviations from this picture of absolute specificity have been observed in the tolerated modifications of both glycosyl donors and acceptors. Additionally, studies toward the design of inhibitors of glycoprotein biosynthesis[205] have also shown that the specificities of glycosyltransferases are not absolute.

Galactosyltransferase (GalT). Because of its availability, β1,4-GalT (E.C. 2.4.1.22)[146, 147] is one of the most extensively studied mammalian glycosyltransferases with regard to synthesis and substrate specificity. The X-ray crystal structure of the bovine enzyme has recently been reported[148]. β1,4-GalT catalyzes the transfer of galactose from UDP-Gal to the 4-position of β-linked GlcNAc residues to produce the Galβ1,4GlcNAc (LacNAc) structure. In the presence of lactalbumin, however, both α- and β-linked substrates are allowed, and glucose is the preferred acceptor. β1,4-GalT has been employed in the *in vitro* syntheses of LacNAc and glycosides thereof, as well as other galactosides[157] (Table 11.3-1).

β1,4-GalT also tolerates 2-deoxyglucose, D-xylose, 5-thioglucose, *N*-acetylmuramic acid, and myoinositol as acceptor substrates[147]. Modifications at the 3- or 6-position of GlcNAc are also accepted. For example, Fucα1,6GlcNAc and NeuAcα2,6GlcNAc are substrates[150]. Acceptor substrates which are derivatized at the 3-position include 3-*O*-methyl-GlcNAc[150], 3-deoxy-GlcNAc, 3-*O*-allyl-GlcNAcβOBu, and 3-oxo-GlcNAc[158]. D-Mannose, D-allose, D-galactose, D-ribose, and D-xylose do not serve as substrates. Monosaccharides which have a negative charge, such as glucuronic acid and α-glucose-1-phosphate, are also not accepted. Fig. 11.3-13 illustrates several disaccharides which can be synthesized with β1,4-GalT[147]. A particulary interesting

Table 11.3-1. Products of galactosyltransferase reactions.

UDP-Gal (or analogs) + GalT	Scale[a]	Ref.
Galβ1,4Glc	C	[147]
Galβ1,4GlcNAc	A	[142, 145]
Galβ1,4GlcNAc-Agarose	C	[147]
Galβ1,4GlcNAc-hexylamine	C	[147]
Galβ1,4GlcNAcβ1,4Gal	C	[147]
Galβ1,4GlcNAcβ1,6Gal	C	[150]
Galβ1,4GlcNAcβ1,3Gal	C	[150]
Galβ1,4GlcβOCH$_2$C$_6$H$_4$(NO$_2$)-CONH-Polymer	D	[151]
Galβ1,4Glcβ1,4GlcβOCH$_2$C$_6$H$_4$(NO$_2$)-CONH-Polymer	D	[151]
Galβ1,4Glcβ1,4GlcβOCH$_2$NH-L-Phe-CONH-Polymer	D	[151]
Galβ1,4GlcNAcβ1,3(Galβ1,4GlcNAcβ1,6)Galβ1,4GlcβOMe	C	[152]
Galβ1,4GlcNAcβ1,6(GlcNAcβ1,3)Galβ1,4GlcβOMe	C	[152]
Galβ1,4(Fucα1,6)GlcNAcβO(CH$_2$)$_8$CO$_2$Me	D	[150]
Galβ1,4(NeuAc(OMe)α2,6)GlcNAcβO(CH$_2$)$_8$CO$_2$Me	D	[150]
Galβ1,4GlcNAcβR; R = *N*-Ac-Asn(OMe)	C	[153]
Galβ1,4GlcNAcβ1,4GlcNAc	C	[153]
Galβ1,4GlcNAcβ1,4GlcNAcβR; R = *N*-Ac-Asn(OMe)	C	[153]
Galβ1,4GlcNAcβO(CH$_2$)$_8$CO$_2$Me	D	[154]
GalNAcβ1,4GlcNAcβO(CH$_2$)$_8$CO$_2$Me	D	[154]
GalNAcβ1,4GlcNAcβ1,2ManβO(CH$_2$)$_8$CO$_2$Me	D	[154]
GalNAcβ1,4GlcNAcβ1,2Manα1,6(GalNAcβ1,4GlcNAcβ1,2Manα1,3) ManβO(CH$_2$)$_8$CO$_2$Me	D	[154]
GlcNAcβ1,4GlcNAcβO(CH$_2$)$_8$CO$_2$Me	D	[154]
Galβ1,4GlcNAcβR; R = GlyGlyAsnGlyGly or *N*-Alloc-PheAsnSerThrIle	C	[155]
Galβ1,3Galβ1,4Glc	D	[156]
Galα1,3Galβ1,4GlcNAc	D	[156]

a A, > 1 g; B, 0.1–1 g; C, 10–100 mg; D, < 10 mg

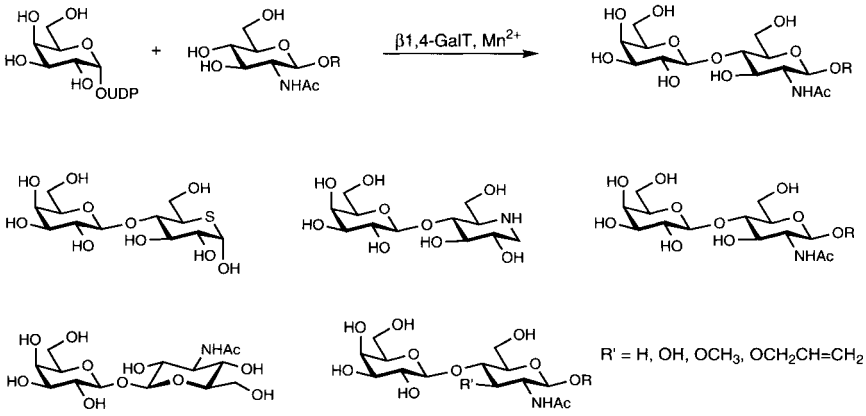


Figure 11.3-13.

example is the β,β-1,1-linked disaccharide, in which the anomeric hydroxyl of 3-acetamido-3-deoxyglucose serves as the acceptor moiety[159]. The acetamido function apparently controls the position of glycosylation.

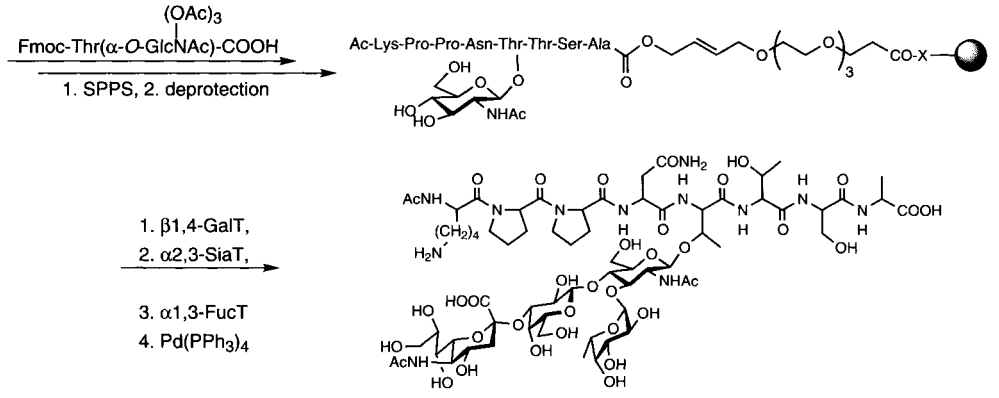


Figure 11.3-14.

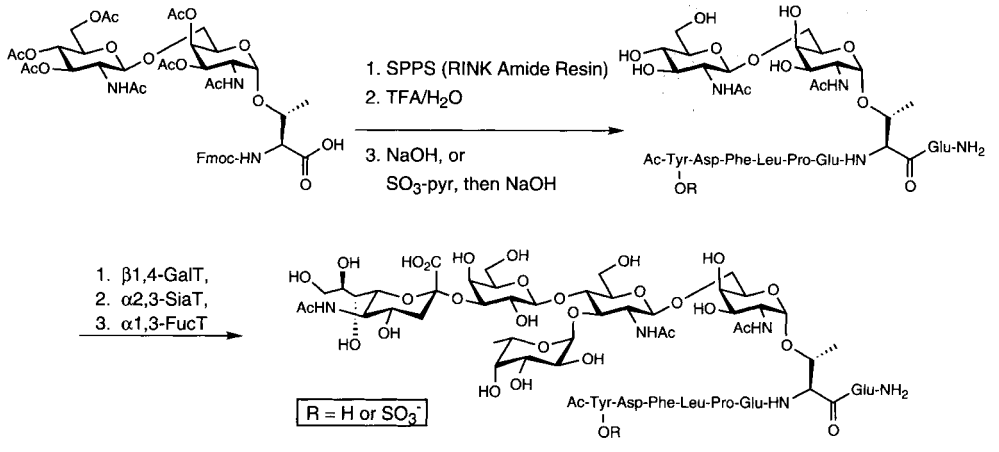


Figure 11.3-15.

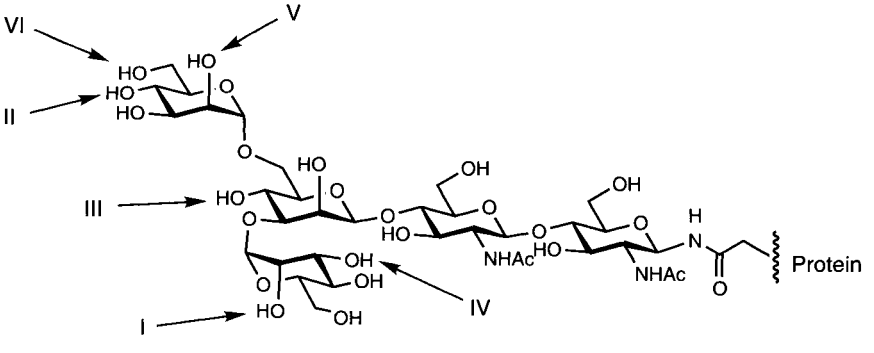
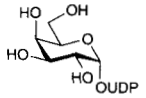
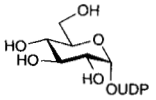
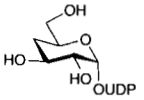
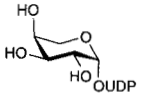
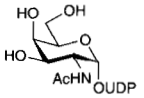
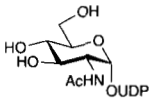
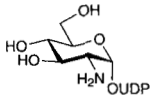
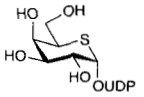


Figure 11.3-16.

Table 11.3-2. Relative rates of b1,4-GalT catalyzed transfer of donor substrates.

Donor substrate		Relative Rate	Ref.
UDP-Gal		100	[165]
UDP-Glc		0.3	[165, 154]
UDP-4-deoxy-Glc		5.5	[165]
UDP-Ara		4	[165]
UDP-GalNAc		4	[154]
UDP-GlcNAc		0	[154]
UDP-GlcN		0.09	[154]
UDP-5-thio-Gal		5	[159]

β1,4-GalT has also been employed in solid-phase oligosaccharide synthesis, and has been used to galactosylate *gluco* and *cellobio* subunits of polymer-supported oligosaccharides and polysaccharides[160]. The resulting oligosaccharides can then be removed from the support by either a photochemical cleavage or a chymotrypsin-mediated hydrolysis. GlcNAc-amino acids and peptides have also been used as substrates for β1,4-GalT to afford galactosylated glycopeptides (Fig. 11.3-16)[153, 155, 160, 161]. The carbohydrate chain can then be further extended with other glycosyltransferases, such as SiaT and FucT[153, 155, 160, 161], as was shown in the enzymatic solid-phase synthesis of glycopeptides from MAdCAM-1 (Fig. 11.3-14)[161b]. Furthermore, solid- and solution-phase techniques can be employed

together for the synthesis of complex sulfated glycopeptides such as those from PSGL-1 (Fig. 11.3-15)[161d]. In terms of glycolipids, β1,4-GalT was utilized in the preparation of a ceramide-linked LacNAc glycoside that was further enzymatically sialylated to provide a GM_3 analog[155, 162].

With regard to the donor substrate, β1,4-GalT also transfers glucose, 4-deoxygalactose, arabinose, glucosamine, galactosamine, GalNAc, and 2-deoxyglucose from their respective UDP-derivatives. This flexibility provides an enzymatic route to oligosaccharides which terminate in β1,4-linked residues other than galactose[326], such as 5-thiogalactose[164] (Table 11.3-2). Although the rate of enzyme-catalyzed transfer for many of these unnatural donor substrates is quite slow, this method is useful for milligram-scale synthesis. Besides β1,4-GalT, other GalTs are also of interest synthetically. Recently, α1,3-GalT has received a heightened focus because of its role in xenotransplantation studies. Several studies of substrate specificity and synthetic potential have also been carried out[164].

Sialyltransferase (SiaT). α2,6- and α2,3-sialyltransferase have been used for oligosaccharide synthesis[166–168]. Sialyltransferases generally transfer *N*-acetylneuraminic acid (NeuAc) to either the 3- or 6-position of terminal Gal or GalNAc residues

Table 11.3-3. Products of sialytransferase reactions.

CMP-NeuAc + α2,6-SiaT	Scale[a]	Ref.
NeuAcα2,6GalβOMe	D	[166]
NeuAcα2,6Galβ1,4GlcβOMe	D	[166]
NeuAcα2,6Galβ1,4GlcNAc	C	[166, 167, 173, 109, 9]
NeuAcα2,6Galβ1,4GlcNAcβOMe	C	[166]
NeuAcα2,6Galβ1,4GlcNAcβ1,3Galβ1,4Glc	C	[166]
NeuAcα2,6Galβ1,4GlcNAcβ1-*N*-Asn	C	[160]
NeuAcα2,6Galβ1,4GlcNAcβ1,2ManαOMe	C	[174]
NeuAcα2,6Galβ1,4GlcNAcβ1,3(Galβ1,4GlcNAcβ1,6)Galβ1,4GlcβOMe	C	[174]
NeuAc(9-*O*-Ac)α2,6Galβ1,4GlcNAc	C	[168]
NeuAcα2,6Galβ1,4GlcNAcβR; R = OH, NH_3, GlyGlyAsnGlyGly or *N*-Alloc-PheAsnSerThrIle	C	[155]
NeuAcα2,6Galβ1,4GlcNAcβ1,4(NeuAcα2,6Galβ1,4GlcNAcβ1,2/3)GalβO$(CH_2)_5CO_2Me$	D	[175]
CMP-NeuAc + α2,3-SiaT		
NeuAcα2,3Galβ1,4GlcβOMe	D	[166]
NeuAcα2,3Galβ1,4GlcNAcβOMe	D	[166]
NeuAcα2,3Galβ1,3GlcNAcβOR; R = Me, Ph, $(CH_2)_5CO_2Me$	D	[166]
NeuAcα2,3Galβ1,3GlcNAcβ1,3Galβ1,4Glc	D	[166]
NeuAcα2,3Galβ1,3GlcNAcβ1,3GalβO$(CH_2)_8CO_2Me$	D	[254]
NeuAcα2,3Galβ1,3GlcNAcβ1,6GalβO$(CH_2)_8CO_2Me$	D	[254]
NeuAcα2,3Galβ1,3GlcNAcβOR (R = Et)	C	[177]
(R = H, $(CH_2)_5CO_2Me$)	D	[167]
NeuAcα2,3Galβ1,3(NeuAcα2,6)GalNAcβOPh	D	[178]
3-*O*-Me-Galβ1,4Glcβ1,6(NeuAcα2,3Galβ1,4)GlcNAcβ1,3Galβ1,4Glcβ1,6-(NeuAcα2,3Galβ1,4)GlcNAcβOMe	D	[179]

a A, > 1 g; B, 0.1–1 g; C, 10–100 mg; D, < 10 mg

(Table 11.3-3)[169]. Some SiaTs accept CMP-NeuAc analogs which are derivatized at the 9-position with amino, fluoro, azido, acetamido, or benzamido groups[139, 140, 142c,d, 168–170]. Azido-, phthalimido-, carbamate, and pivaloyl analogs of LacNAc and Galβ1,3GalNAc are also substrates for the enzymes[172]. Sialyltransferases have been used to append NeuAc to galactose on the terminus of glycopeptides[161], glycolipids[180], and glycoproteins[181].

Fucosyltransferase (FucT). Fucosyltransferases are involved in the biosynthesis of many blood-group substances and tumor-associated antigens. α1,3-FucT L-fucosylates the GlcNAc 3-position of LacNAc and sialylα2,3LacNAc to provide the Lewis X and sialyl Lewis X structures, respectively[127]. Several other acceptor substrates with modifications in the GlcNAc residue [lactose, Galβ1,4Glucal, Galβ1,4(5-thioGlc)] can also be fucosylated by various FucT isozymes (Table 11.3-4)[182]. α-1,3/4-FucT fucosylates either the GlcNAc 3-position of Galβ1,4GlcNAc or the GlcNAc 4-position of Galβ1,3GlcNAc (as well as the sialylated versions) to afford (sialyl)Lewis X or (sialyl)Lewis A, respectively[174, 175, 183]. Furthermore, α1,3/4-FucT will transfer a fucose residue which is substituted on C-6 by a very sterically demanding structure. Notably, a synthetic blood group antigen can be attached, and the resulting "oligosaccharide" can be transferred to an acceptor from its GDP derivative[186]. This approach has been employed to alter the antigenic properties of cell-surface glycoproteins.

The Lewis A α1,4-FucT has been used to transfer unnatural fucose derivatives- from their GDP esters. 3-Deoxyfucose and L-arabinose are transferred to LacNAc-$\beta O(CH_2)_8CO_2CH_3$ at a rate of 2.3% and 5.9%, respectively, relative to L-fucose[185]. Moreover, α1,3-FucTs have been extensively employed as the final step in an enzymatic cascade for the synthesis of complex oligosaccharides[187], glycopeptides[161], and glycoproteins[181] in which the sLe^x structure is formed. *N-Acetylglucosaminyltransferase (GlcNAcT). In vivo,* the *N*-acetylglucosaminyl transferases control the branching pattern of *N*-linked glycans[188, 189]. Each of these enzymes transfers a β-GlcNAc residue from UDP-GlcNAc to a high mannose-based acceptor. The GlcNAc transferases I-VI, which catalyze the additon of the GlcNAc residues to

Table 11.3-4. Products of fucosyltransferase reactions.

GDP-Fuc + α1,2 or α1,3/4 FucT	Scale[a]	Ref.
Fucα1,2GalβOR; R = CH_2CH_3, $(CH_2)_6NH_2$	C	[184]
Fucα1,2Galβ1,4GlcNAcβOR; R = H, $(CH_2)_6NH_2$	C	[184]
Fucα1,3(NeuAcα2,3Galβ1,4)GlcNAcβ$O(CH_2)_5CO_2Me$	C, D	[175, 183]
Fucα1,3(Galβ1,4)-5-thio-Glc	C	[183]
Fucα1,4(Galβ1,3)GlcNAc	C	[183]
Fucα1,3(NeuAcα2,3Galβ1,4)Glucal	D	[163]
Fucα1,4(NeuAcα2,3Galβ1,3)GlcNAcβ1,6GalβO$(CH_2)_5CO_2Me$	D	[185]
Fucα1,4(NeuAcα2,3Galβ1,3)GlcNAcβ1,3GalβO$(CH_2)_5CO_2Me$	D	[185]
Fucα1,4(Galβ1,3)GlcNAcβ$O(CH_2)_8CO_2Me$	D	[185]
3-deoxy-Fucα1,4(Galβ1,3)GlcNAcβ$O(CH_2)_8CO_2Me$	D	[184]
5-desmethyl-Fucα1,4(Galβ1,3)GlcNAcβ$O(CH_2)_8CO_2Me$	D	[185]

a A, > 1 g; B, 0.1–1 g; C, 10–100 mg; D, < 10 mg

Table 11.3-5. Products of GlcNAc-transferase reactions.

UDP-GlcNAc (or analogs) + GlcNAcTase	Scale[a]	Ref.
UDP-GlcNAc + GlcNAcT I		
GlcNAcβ1,2Manα1,3(Manα1,6)ManβO$(CH_2)_8CO_2Me$	C	[59, 192]
3-deoxy, 4-deoxy, or 6-deoxy-GlcNAcβ1,2Manα1,3(Manα1,6)ManβO $(CH_2)_8CO_2Me$	D	[190]
UDP-GlcNAc + GlcNAcT II		
GlcNAcβ1,2Manα1,6(GlcNAcβ1,2Manα1,3)ManβO$(CH_{r2})_8CO_2Me$	D	[192]
UDP-GlcNAc + GlcNAcT		
GlcNAcβ1,6(Galβ1,3)GlcNAc	D	[193]
UDP-Glc + GlcT		
GlcβOR; R = CH_2CH_3, $(CH_2)_6NH_2$	C, D	[194]

a A, > 1 g; B, 0.1–1 g; C, 10–100 mg; D, < 10 mg

the core Asn-linked pentasaccharide of glycoproteins (Fig. 11.3-16), have been identified and characterized[188, 189].

GlcNAc transferases have been utilized for the synthesis of natural and non-natural oligosaccharides (Table 11.3-5). In addition to transferring GlcNAc, GlcNAcT I from human milk catalyzes the transfer of 3-, 4-, or 6-deoxy-GlcNAc from its respective UDP derivative to Man α1,3(Manα1,6)ManβO$(CH_2)_8CO_2CH_3$[190, 196]. The 4- and 6-deoxy-GlcNAc analogs can also be transferred by GlcNAcT II, although UDP-3-deoxy-GlcNAc is not a substrate for this enzyme[190]. The core 2 GlcNAcT can employ UDP-trifluoro-GlcNAc as a substrate[195]. GlcNAcT has also been used to attach the terminal GlcNAc of GlcNAcβ1,4GlcNAcα dolichyl pyrophosphate, a substrate of oligosaccharyltransferase[191].*Mannosyltransferase (ManT).* Various mannosyltransferases have been shown to transfer mannose and 4-deoxymannose from their respective GDP adducts to acceptors[197]. α1,2-ManT transfers mannose to various derivatized α-mannosides and α-mannosyl peptides to produce the Manα1,2Man structural unit[198]. This method has also been extended to whole cells as a source of α1,2-ManT[199]. Mannosyltransferases from pig liver accept GlcNAcβ1,4GlcNAc phytanyl pyrophosphate, an analog of the natural substrate in which the phytanyl moiety replaces dolichol[200]. Overexpression of β1,4-ManT has also been instrumental in the synthesis of an *N*-glycan core structure[201] as well as the bacterial O antigen[202]. β1,4-ManT is especially valuable synthetically, as β-mannosyl glycosides are exceedingly difficult to form chemically.

Sucrose synthetase. The fructose derivatives 1-azido-1-deoxy-, 1-fluoro-1-deoxy-, 6-deoxy-, 6-fluoro-6-deoxy-, and 4-fluoro-4-deoxy-fructose have been used as glycosyl acceptors in the sucrose synthetase-catalyzed synthesis of sucrose analogs[203]. 6-Deoxy- and 6-fluoro-6-deoxy-fructose were generated *in situ* from the corresponding glucose derivatives under catalysis by glucose isomerase[203]. Sucrose synthetase has also been extensively employed in the synthesis of nucleotide sugars[204].

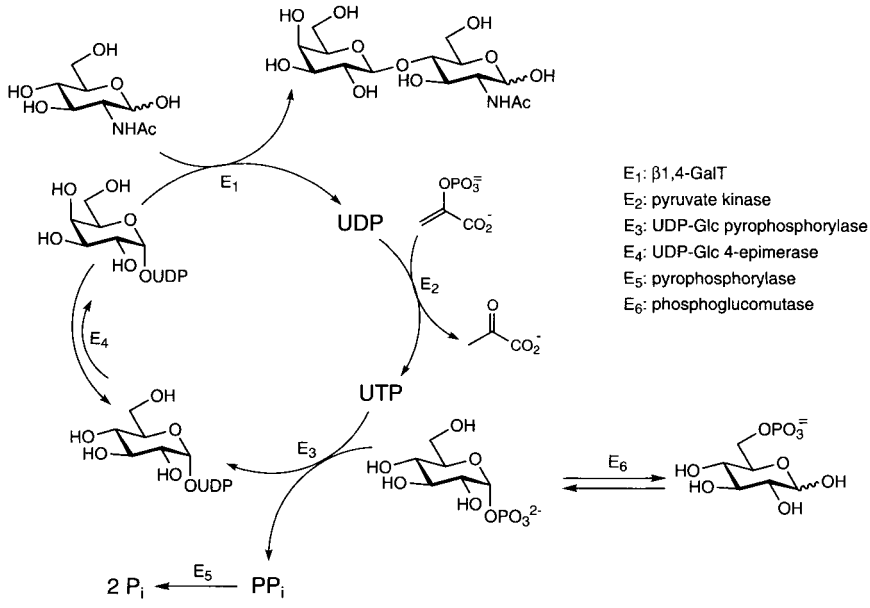


Figure 11.3-17.

11.3.2.3
In Situ Cofactor Regeneration

Though analytical and small-scale synthesis using glycosyltransferases is extremely powerful, the high cost of sugar nucleotides and the product inhibition caused by the released NMP or NDP present major obstacles to large-scale synthesis. A simple solution to both of these problems is to regenerate the sugar nucleotide *in situ* from the released NDP[205]. The first example of this strategy was the β1,4-GalT-catalyzed synthesis of LacNAc[201] (Fig. 11.3-17). Only a catalytic amount of UDP-Gal is initially used to glycosylate GlcNAc. However, UDP-Gal is regenerated from the product UDP and galactose using an enzyme-catalyzed reaction sequence which requires stoichiometric amounts of a phosphorylating agent. Several oligosaccharides have been prepared using routes based on this concept[150]. Another regeneration system for UDP-Gal, which is based on the use of galactose-1-phosphate uridyltransferase, has also been developed[206]. A third, which employs sucrose synthetase for recycling of UDP-Glc/UDP-Gal from sucrose and UMP has recently been described[207]. A very recent approach to recycling systems employs coupling metabolically engineered bacterial cells for large scale sugar nucleotide production, to date including UDP-Gal (Fig. 11.3-18)[209] and CMP-NeuAc[144].

In situ cofactor regeneration offers several advantages. First, a catalytic amount of NDP and a stoichiometric amount of monosaccharide are used as starting materials rather than a stoichiometric quantity of sugar nucleotide, thus tremendously reducing costs. Second, product inhibition by the released NDP is minimized

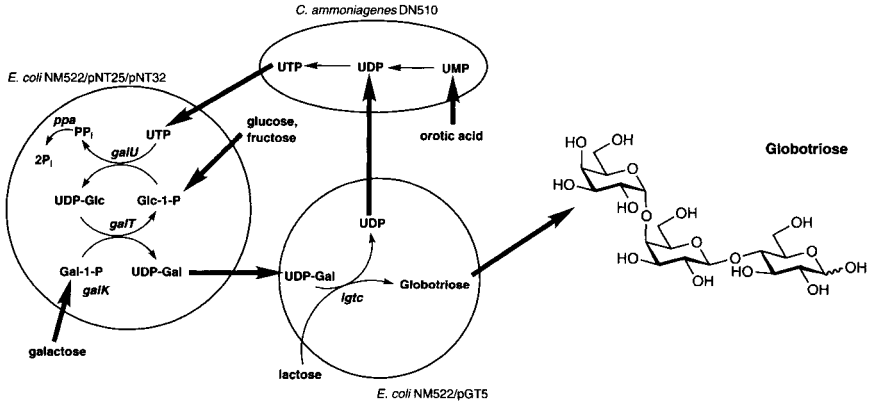
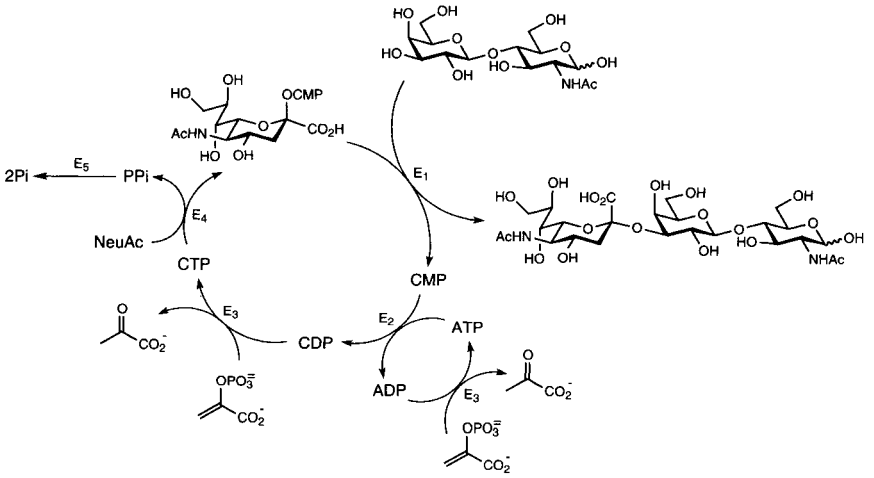


Figure 11.3-18.

because of its low concentration in solution. And third, isolation of the product is greatly facilitated.

A multi-enzyme regeneration system for CMP-NeuAc is illustrated in Fig. 11.3-19[135, 208]. This system follows the same basic principles as the UDP-Gal recycling system. A CMP-NeuAc synthetase/α2,3-SiaT fusion enzyme with increased stability has also been applied to this procedure[210]. The development of these regeneration systems, as well as those for GDP-Man[198], GDP-Fuc[127], and UDP-GlcUA[130] should facilitate the widespread use of glycosyltransferases for oligosaccharide synthesis. Notably, when UDP-GlcUA and UDP-GlcNAc recycling systems are combined with hyaluronic acid synthase, HA polymers can be produced[211]. New



E_1: α2,3-sialyltransferase; E_2: nucleoside monophosphate kinase;
E_3: pyruvate kinase; E_4: CMP-NeuAc synthetase; E_5: pyrophosphatase

Figure 11.3-19.

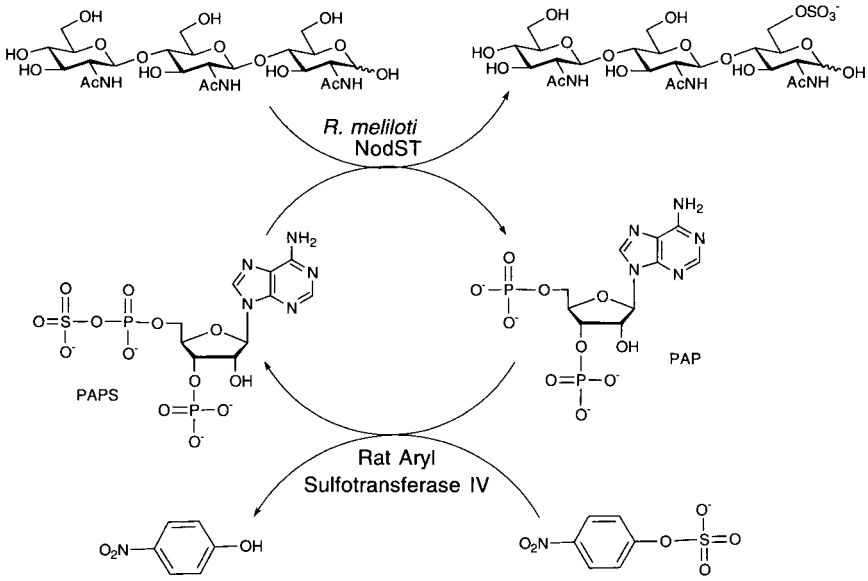


Figure 11.3-20.

systems for the recycling of PAPS for the synthesis of complex sulfated carbohydrates have also recently been developed (Fig. 11.3-20) [212].

11.3.2.4

Cloning and Expression of Glycosyltransferases

While many glycosyltransferases catalyze similar reactions and use the same donor substrate, there appears to be little sequence homology among the different enzymes of this class (i.e GalT vs SiaT, etc.). There is, however, a significant cross species homology for the same glycosyltransferase. For instance, one finds 86% identity when comparing the β1,4-GalT protein sequence from humans to that from rat. The different glycosyltransferases do exhibit some similarity in that their cDNA sequences encode regions consistent with a short *N*-terminal tail, a hydrophobic transmembrane sequence, a short stem sequence, and a large *C*-terminal catalytic domain [213]. In addition to the membrane-bound form of the glycosyltransferases, soluble enzymatic forms have also been identified in body fluids such as blood, milk, and colostrum. Indeed, these fluids have been sources for the purification of specific glycosyltransferases [214–217]. A comparison of the cDNA sequences of these soluble enzymes with full-length glycosyltransferase genes suggests that the stem region has been cleaved to release the large catalytic domain from the membrane. Presumably, this theme of signal sequence cleavage is consistent for all the glycosyltransferases (Fig. 11.3-21) [219].

The amount of a glycosyltransferase that can be isolated from natural sources is often limited by the low concentrations of these enzymes present in most tissues and

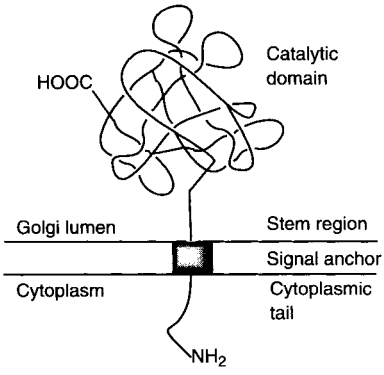


Figure 11.3-21.

body fluids. The purification of glycosyltransferases is further complicated by their relative instability[61]. For this reason, a great deal of interest has been directed toward the cloning of glycosyltransferase genes into convenient expression systems[43, 219] (Table 11.3-6). The general strategy involved is outlined in Fig. 11.3-22. The glycosyltransferase gene must first be identified and isolated from the mRNA pool via the cloning of the cDNA to make a cDNA library. This library is then screened to identify the glycosyltransferase gene of interest among ~ 10^6 different sequences present. Once identified, the gene is sequenced and a more complete cloning strategy is developed in order to incorporate the gene into an expression vector. This laborious path has been successfully employed by several groups, many of whom are referenced in Table 11.3-6. The nuances to the general cloning scheme used by these groups are discussed below.

Among the organs that have been used for the isolation of glycosyltransferase mRNA are the liver[220, 221], placenta[222], mammary gland[223], testis[224], and

Table 11.3-6. Cloned glycosyltransferases of the glycoprotein and glycolipid pathways.

Enzyme	Source	Ref.
UDP-Glucuronosyltransferase	murine liver	[220]
	rat liver	[221]
Mannosyltransferase	yeast	[232]
α2,6-Sialyltransferase	rat liver	[229]
α2,3-Sialyltransferase	porcine submaxillary gland	[233]
β1,4-Galactosyltransferase	bovine placenta	[222]
	bovine mammary gland	[223]
	murine mammary gland	[234]
	bovine liver	[235]
	murine F9 cells	[236]
	bovine kidney epithelial cells	[237]
	murine testes	[224]
	human placenta	[238]
β1,3-Galactosyltransferase	calf thymus	[225]
	murine F9 cells	[226]
α1,2-Fucosyltransferase	human A431 cells	[227]
α1,3/4-Fucosyltransferase	human A431 cells	[239]

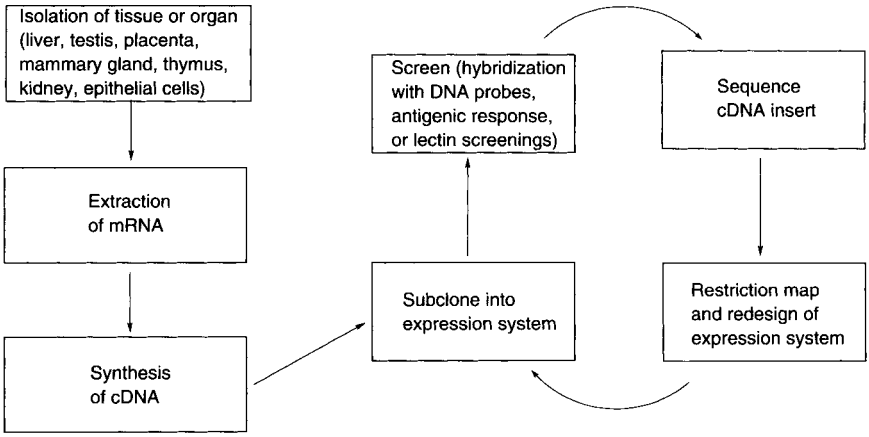


Figure 11.3-22.

thymus[225]. In addition, tissue cultures have been used in place of the organ[226, 227]. From these sources, cDNA is synthesized, and the double stranded cDNA is ligated in λ phage via a convenient linker and packed into bacteriophages. The bacteriophages are then plated onto a lawn of *E. coli*, and screened for the desired gene or gene product. Identification of the glycosyltransferase gene has most frequently been achieved by the hybridization of the gene to specific radiolabeled DNA probes[220–223]. Screening in this manner obviously requires a previous knowledge of the gene sequence – information that in some cases may be obtained by extrapolation from a partial protein sequence or from the DNA sequence of the glycosyltransferase from a related source. Two other approaches have been used to screen glycosyltransferase cDNA libraries, both requiring successful transcription and translation of the gene product. In the cloning of the α2,6-SiaT from rat liver, Weinstein et al. used polyclonal antibodies raised to the purified enzyme to screen the λ plaques[228]. The approach used by Larsen et al. alleviated the need for a previous knowledge of the sequence[226]. This method made use of the specificity of a lectin that recognizes the surface-expressed glycoconjugate product of α1,3-GalT. The transfected cells were then panned in dishes coated with the lectin. The adherent cells were isolated and re-panned for further purification. Each of these techniques makes use of libraries in which there are very few copies of the desired gene. A greater chance of success may be possible if the number of copies of the genes could be amplified. The introduction, in 1985, of an *in vitro* amplification method based on the polymerase chain reaction (PCR) fulfilled this need[229, 230]. Of course PCR (and ECPCR)[230], like the hybridization screening, requires a specific knowledge of the sequence.

Once identified, the genes are sequenced using standard procedures. Recloning of the gene into an expression vector is then used to develop an expression system. This recloning has been performed on only a few of the glycosyltransferases. Toghrol et al. have inserted the mouse liver GlcUAT gene into the yeast vector pEVP11 and expressed the enzyme in *Saccharomyces cerevisiae*[220]. The rat liver GlcUAT, on the

other hand, has been expressed in COS cells using the SV40 vector[221]. Expression in COS cells using SV40 was also applied to the cloning of bovine β1,4-GalT[222]. A noteworthy approach toward the expression of glycosyltransferases in *E. coli* has been developed by Aoki et al. to obtain human β1,4-GalT[231]. A unique *Rsr*II restriction site in the β1,4-GalT gene allowed the dissection of the sequence at the location of signal peptidase cleavage. The cohesive terminus was digested with Klenow fragment, and the blunt end ligated to pINIII-ompA$_2$[232] at a Klenow fragment treated *Eco*RI site. This generated the code for a soluble fusion protein of β1,4-GalT with the ompA signal sequence. Transcription and translation of this sequence in *E. coli* produced an active enzyme that was released into the periplasmic space. Purification and *N*-terminal sequencing of the enzyme verified the expression of the soluble form of β1,4-GalT with an additional tripeptide *N*-terminal tail. The kinetic parameters of this enzyme appear to be identical to the isolated native enzyme.

To date, over a hundred glycosyltransferases have been cloned[21]. Expression and production in quantities sufficient for enzymatic synthesis is, however, another matter. Only a handful of glycosyltransferases are currently commercially available. Given the advantages of enzymatic synthesis of oligosaccharides over traditional schemes, research into the overexpression of glycosyltransferases will undoubtedly continue to be developed.

11.3.3
Non-Leloir Glycosyltransferases: Transfer of Glycosyl donors from Glycosyl Phosphates and Glycosides

Oligosaccharides can also be prepared using non-Leloir glycosyltransferases. Phosphorolysis is reversibly catalyzed by glucan phosphorylases for the synthesis of polysaccharides. For example, sucrose[240a] and trehalose[240b] have been synthesized by the corresponding phosphorylase[240a]. Sucrose phosphorylase has also been used in the recycling of UDP-Gal[241]. Other enzymes of this class are involved in the synthesis of dextrans and levans[242].

Modified polysaccharides may provide materials with more desirable physical and biological properties than their natural counterparts. Approaches to controlling glycopolymer characteristics have included the control of genes encoding the enzymes responsible for their production, regulation of the activity of these enzymes, or the influence of their *in vitro* synthesis[243]. Potato phosphorylase has been used *in vitro* to prepare maltose oligomers,[99] as well as a family of linear, star, and comb-shaped polymers[244]. This enzyme will synthesize polysaccharides in the presence of primers[99].

A coupled potato phophorylase/sucrose phosphorylase system, where glucose-1-phosphate is generated *in situ* from sucrose and inorganic phosphate, has been employed for polysaccharide synthesis[100]. The inorganic phosphate liberated by potato phosphorylase is used by sucrose phosphorylase to drive the formation of polymer, thereby increasing the yield. Regulation of the molecular weight of the polysaccharide product can be controlled by the concentration of the primer.

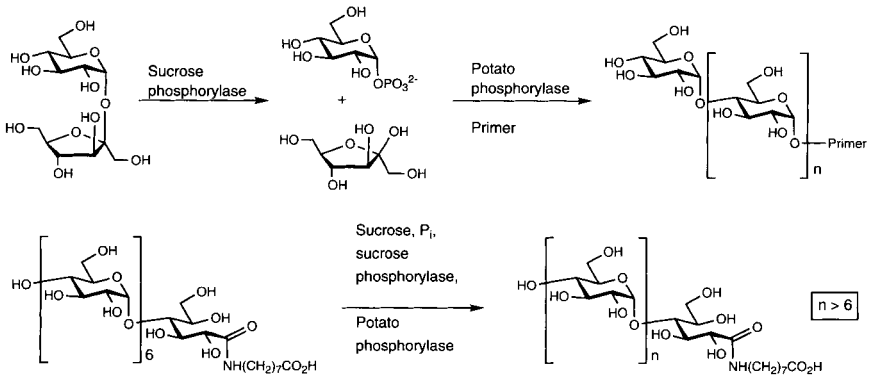


Figure 11.3-23.

Unnatural primers bearing functional groups can also be used to prepare tailor-made polysaccharides for further manipulation, e.g. attachment to protein or other compounds (Fig. 11.3-23).

Cyclodextrin α1,4-glucosyltransferase (CD α1,4-GlcT, E.C. 2.4.1.19) from *Bacillus macerans* catalyzes the cyclization of oligomaltose to form α-, β- and δ-cyclodextrin, and the transfer of sugars from cyclodextrin to an acceptor to form oligosaccharides [245, 246]. This enzyme can transform α-glucosyl fluoride into a mixture of α- and β-cyclodextrins and malto-oligomers [247]. When immobilized on a silica gel support, CD α1,4-GlcT was very stable, with no loss of activity observed after 4 weeks when stored at 4 °C. This type of enzymatic catalysis may provide a new route to unnatural cyclodextrin analogs and novel oligosaccharides, as glucose analogs are also substrates. For example, oligoglucosyl deoxynojirimycin and *N*-substituted derivatives were produced under CD α1,4-GlcT catalysis. Subsequent hydrolysis by glucoamylase gave glycosylazasugars like 4-*O*-α-D-glucopyranosyl deoxynojirimycin in ~60% yield (Fig. 11.3-24) [248] The *N*-methyl derivative was reported to be a potent inhibitor of glucosidase.

In spite of the progress that has been made, several difficulties limit the use of cell-free enzymes for the synthesis of polysaccharides. The major problem is the complexity of many polysaccharide-synthesizing systems. Isolation, purification, and stabilization of the required enzymes is often difficult, as many enzymes lose activity when they are no longer membrane-associated. Enzyme isolation from eukaryotic sources is tedious, because of low cellular enzyme concentration. It is unlikely that cell-free enzymatic synthesis will provide better routes to most natural polysaccharides than do fermentation and isolation. The use of genetic engineering,

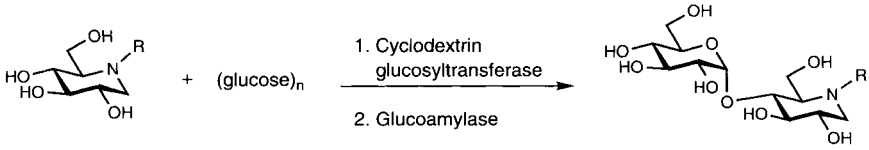


Figure 11.3-24.

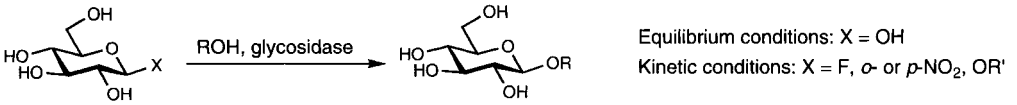


Figure 11.3-25.

both using classical genetics and recombinant DNA technology, is an approach being used to prepare modified carbohydrate polymers[250].

11.3.4
Glycosidases

Glycosidases catalyze the hydrolysis of glycosidic linkages, typically with retention of configuration at the anomeric carbon (β-galactosidase and lysozyme), but sometimes with inversion (trehalase and β-amylase)[251]. Enzymatic hydrolysis is thought to be mechanistically similar to acid-catalyzed hydrolysis of glycosides. Both proceed via an oxonium ion intermediate or a transition state having oxonium character[251]. A proximal carboxylate in the enzyme active site appears as a common structural motif among glycosidases, and presumably acts to stabilize this intermediate or transition state. Whether the oxocarbenium ion exists as a stabilized ion pair or is trapped by the carboxylate to form a glycosyl ester has been the subject of debate. However, an α-glycosyl enzyme intermediate has been observed by ^{19}F NMR in the β-glucosidase-catalyzed hydrolysis of 2-deoxy-2-fluoro-D-glucosyl fluoride, and was shown to be a catalytically competent species[252].

Glycosidase-catalyzed glycoside synthesis is quite analogous to protease-catalyzed peptide synthesis. As with proteases, glycosidases may be used under either equilibrium or kinetically controlled conditions for synthetic purposes (Fig. 11.3-25)[84, 253].

11.3.4.1
Equilibrium-controlled Synthesis

The obvious approach to glycosidase catalyzed synthesis of glycosidic linkages involves reversing the catabolic role of the enzyme. Indeed, examples of equilibrium-controlled synthesis were reported by Bourquelot at the early part of this century[253]. Synthesis by this approach involves an endergonic process with the free energy change under ambient aqueous conditions favoring bond cleavage by approximately 4 kcal/mol. Reaction conditions must therefore be manipulated in order to drive the reaction to produce glycoside.

In efforts to shift the equilibrium toward product, the addition of water-miscible organic cosolvents was investigated, but this usually results in enzyme inactivation and a decrease in K_m for the glycoside acceptor[254]. The use of high substrate concentrations and elevated reaction temperatures have also been explored. Johansson et al.[255] reported the synthesis of mannose disaccharides with jack bean α-mannosidase, while Ajisaka et al.[256] utilized almond β-glucosidase with glucose

concentrations as high as 90% w/v to obtain glucose disaccharides. Carbon-celite[257] and active carbon columns have been developed as molecular traps which selectively absorb the product as the reaction mixture is circulated through the column. Yields, however, are still only about 15%. Though quite simple in theory, the equilibrium approach generally provides poor yields and the formation of side products, which make purification difficult.

11.3.4.2
Kinetically Controlled Synthesis

Kinetically controlled synthesis relies on the trapping of a reactive intermediate generated from an activated glycosyl donor to form a new glycosidic bond[84, 254]. The trapping agent is generally an exogenous nucleophile. Suitable glycosyl donors for this transglycosylation reaction include di- or oligosaccharides, aryl glycosides, and glycosyl fluorides (Table 11.3-7). The reactive intermediate must be trapped by the glycosyl acceptor more rapidly than by water[258]. Under the proper conditions, glycoside formation may be favored kinetically, but hydrolysis is always favored thermodynamically. The reaction must therefore be carefully monitored, and arrested when the glycosyl donor is consumed in order to minimize subsequent glycoside hydrolysis. Recently, mutant glycosidases have been engineered to avoid competing product hydrolysis. Because these enzymes lack a catalytic nucleophile in the active site, they can synthesize but not hydrolyze glycosides[293g,h].

In a comparative study of kinetically vs thermodynamically controlled synthesis of Galβ1,6GalNAc, the kinetic approach afforded of 10-fold increase in product yield (20% vs 2%)[259]. Yields in kinetically controlled synthesis generally range from 20 to 40%. Although addition of organic solvent has not generally been observed to increase product yields, increase of acceptor or donor concentration seems to be quite effective. As an exception, though, polyethylene glycol-modified β-galactosidase is soluble in organic solvents and seems to be suitable for transglycosylation[260].

The kinetically controlled approach has primarily been applied to the retaining glycosidases. However, using glycosyl fluorides as glycosyl donors, an inverting glycosidase has been used to afford products having the configuration at the anomeric position which is opposite to that of the donor[261]. For example, the α,α-linkage of α-D-glucopyranosyl-α-D-xylopyranoside has been prepared utilizing β-glucosylfluoride and α-trehalase[262].

11.3.4.3
Selectivity

The primary goal of enzymatic glycoside formation or oligosaccharide synthesis is to achieve selectivity which is difficult to achieve by chemical methods. Glycosidase-catalyzed chemoselective reaction of one hydroxyl group of an unprotected sugar with the glycosyl donor has been observed, although the selectivity is not necessarily absolute or predictable. Kinetically controlled synthesis has been more successful

Table 11.3-7. Synthesis of oligosaccharides and other glycosides using glycosidases.

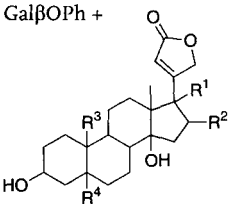
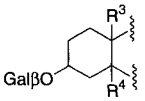
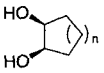
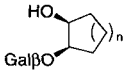
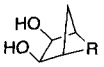
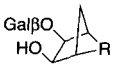
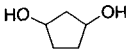
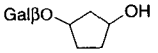
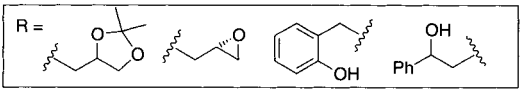
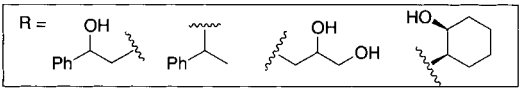
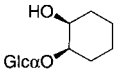
Substrate	Product	Scale[a]	Ref.
α-Galactosidase [252, 462]			
Raffinose + $CH_2 = CHCH_2OH$	GalαO$CH_2CH = CH_2$	A	[263]
GalαO-*p*-$PhNO_2$ + GalαO$CH_2CH = CH_2$	Galα1,3GalαO$CH_2CH = CH_2$	B	[263]
+ Gal(α or β)OMe	Galα1,3/6GalαOMe	B	[263]
GalαO-*o*-$PhNO_2$ + Gal(α or β)O-*p*-$PhNO_2$	Galα1,2/3GalαO-*p*-$PhNO_2$	C	[263]
β-Galactosidase [252, 463]			
GlcβOPh + ROH (R = alkyl)	GlcβOR	C	[267]
Galβ1,4Glc + GlcNAc-R (R = OH or SEt)	Galβ1,3GlcNAcR	B	[265]
Galβ1,4Glc + GlcNAc	Galβ1,4GlcNAc	A	[269]
+ GlcNAc	Galβ1,3-GlcNAc	B	[259, 268]
+ GalNAc	Galβ1,6-GalNAc	B	[259, 268]
+ ROH (R = allyl, benzyl, $TMS(CH_2)_2$)	GalβOR	A, B	[263]
	Galβ1,3/6GalβOR	B	[263]
GalβOPh + 	 1. R^1, R^4 = H, R^2 = OH, R^3 = CH_3 (Gitoxigenin) 2. R^1, R^2, R^4 = H, R^3 = CH_3 (Digitoxigenin) 3. R^1, R^2 = O, R^3 = CH^3, R^4 = H (16β, 17β-epoxy-17α-digitoxigenin) 4. R^1, R^2 = H, R^3 = CHO, R^4 = OH (Strophanthidin)	B	[270]
GalβO-*o*-$PhNO_2$ + GalαOMe	Galβ1,6GalαOMe	C	[263]
+ GalβOMe	Galβ1,6/3GalβOMe	C	[263]
GalβOPh + ROH (R = alkyl)	GalβOR	B	[271]
GlcβOPh + BnOH	GlcβOBn	B	[271]
Galβ1,4Glc + sucrose	Galβ1,6α1,2Fru	E	[272]
Galβ1,4Glc or GalβOPh +			
 n = 1 or 2	 (89–90% de)	E	[273]
 R = $(CH_2)_2$ or CH = CH	 (75% de)	E	[273]
	 (50% de)	E	[273]

Table 11.3-7. (cont.).

Substrate	Product	Scale[a]	Ref.
β-Galactosidase [252, 463]			
Galβ1,4Glc or GalβOPh + ROH	GalβOR	A, B	[274, 275]
			
GlcβOPh + ROH	GlcβOR	B	[275, 153, 154]
			
Galactal + ROH	2-deoxy-GalβOR	E	[159]
Galactal + Galactal	2-deoxy-Galβ1,3/6Galactal + 2-deoxy-Galβ1,3-2-deoxy-Galβ1,6Galactal	C	[160]
GalβO-*o*-$PhNO_2$ + Z-Ser-OR	GalβO-Z-Ser-OR	C	[155]
GalβO-*o*-$PhNO_2$ + Ser	GalβOSer	E	[279]
Galβ1,4Glc + Z-Ser-OMe	GalβO-Z-Ser-OMe	B	[280]
α-Mannosidase [252, 464]			
ManαO-*p*-$PhNO_2$ + ManαOR	Manα1,2/6ManαOR (R = Me or *p*-$PhNO_2$)	B	[263]
α-Glucosidase [252, 465]			
Glc + Fru	Glcα1,1Fru	D	[263]
Glcβ1,4Glc +		C	[154, 267]
β-Glucosidase [252, 465]			
Glc	Glcβ1,4/6Glc	C	[284]
Glcβ1,4Glc	Glcβ1,4Glcβ1,4Glc	C	[284]
β-*N*-Acetylhexosaminidase [252, 466]			
Gal(Glc)NAcβO-*p*-$PhNO_2$ + Glc(NAc)βOMe	Gal(Glc)NAcβ1,3/4Glc(NAc)βOMe	C	[285]
Gal(Glc)NAcβO-*p*-$PhNO_2$ + Glc(NAc)αOMe	Gal(Glc)NAcβ1,4/6Glc(NAc)αOMe	C	[285]
α-Fucosidase [252, 467]			
FucαO-*p*-$PhNO_2$ + Gal(α or β)OMe	Fucα1,3Gal(α or β)OMe	E	[84]
Neuraminidase [252, 468]			
NeuAcα-*p*-$PhNO_2$ + Gal(α or β)OMe	NeuAcα2,3/6Gal(α or β)OMe	D	[289]
NeuAcα-*p*-$PhNO_2$ + Galβ1,4GlcNAc	NeuAcα2,3/6Galβ1,4GlcNAc	D	[289]

a A, > 1 g; B, 0.1–1 g; C, 10–100 mg; D, < 10 mg; E, not reported

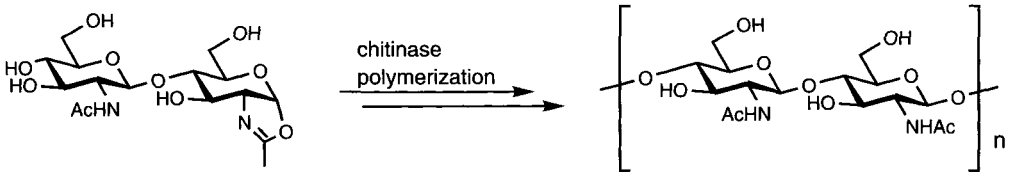


Figure 11.3-26.

than thermodynamically controlled synthesis in achieving selectivity. In general, the primary hydroxyl group of the acceptor reacts preferentially over secondary hydroxyl groups, resulting in a 1,6-glycosidic linkage. Some control of selectivity has been demonstrated by the selection of an appropriate donor/acceptor combination. For example, the α-galactosidase-catalyzed reactions of α-Gal-OPh-*p*-NO_2 with α-Gal-OMe and β-Gal-OMe form predominantly α-1,3 and α-1,6 linkages, respectively[263]. The substituent at the anomeric center of the acceptor controls the position of glycosylation to some extent. αGal-OPh-*p*-NO_2' acting both as donor and acceptor, forms preferentially the α-1,3 linkage, whereas the *ortho*-nitrophenyl glycoside reacts in a similar fashion to form predominantly the α-1,2 linkage[263]. With β-galactosidase, β-1,3-linked disaccharides were formed preferentially when benzyl or allyl β-galactoside was used as acceptor[84, 263]. The use of glycals as acceptors has also been employed as a means of controlling selectivity[264].

One can also use glycosidases from different species to control the regioselectivity. For example, the β-galactosidase from testes catalyzes the formation of Galβ1,3GlcNAc[265] from lactose and GlcNAc. The minor products produced in this preparation were then hydrolyzed by the *E. coli* β-galactosidase, which preferentially hydrolyzes β-1,6-linked galactosyl residues. The overall yield of the β-1,3-linked disaccharides was around 10–20%.

Synthesis of polysaccharides based on kinetically controlled glycosidase reactions have been accomplished, as exemplified by the cellulase-catalyzed reaction of β-cellobiosyl fluoride to form cellulose, with degree of polymerization < 22[266]. In another strategy, employing a chitin hydrolysis transition state analog, chitinase catalyzed polymerization was accomplished without competing hydrolysis (Fig. 11.3-26)[249]. Glycosyl transfer to non-sugar acceptor has also been demonstrated. These reactions are especially interesting with chiral, racemic, or *meso* alcoholic acceptors, as one might expect some degree of diastereoselectivity due to the asymmetric microenvironment of an enzyme active site. Such selectivity has indeed been observed, with diastereoselectivities ranging from moderate to exceptional, as illustrated in Table 11.3-7.

11.3.5
Synthesis of *N*-glycosides

Nucleosides and their derivatives are ubiquitous in nature, and are involved in a myriad of biochemical phenomena, most notably the storage and transfer of genetic information. Interest in this class of compounds has been stimulated by the efficacy

of certain nucleosides as antiparasitic[297] and antiviral agents[298, 299]. Nucleosides have traditionally been prepared by chemical methods[300] requiring multiple protecting group manipulations and glycosyl activation procedures. Problems encountered include control of anomeric configuration and regiospecific C-N glycoside formation when there are several possible nucleophilic groups in the purine or pyrimidine base.

11.3.5.1
Nucleoside Phosphorylase

Enzymatic preparations of both natural and unnatural nucleosides have been reported using nucleoside phosphorylases as catalysts[301]. These enzymes catalyze the reversible (but highly favorable) formation of a purine or pyrimidine nucleoside and inorganic phosphate from ribose-1-phosphate (R-1-P) and a purine or pyrimidine base. Nucleoside synthesis has relied on the transfer of the ribose moiety of a readily available nucleoside to a different purine or pyrimidine base or analogs through the intermediacy of R-1-P. This work has been done primarily with isolated enzymes[302], but whole cells have also been employed in a few cases[303]. The deleterious hydrolases present in whole cells could be largely neutralized by conducting the reactions at 60 °C, a temperature at which the nucleoside phosphorylases maintain < 70% of their activity for 3–5 days[303].

The first synthetic strategy toward nucleosides employed involves isolation of R-1-P, which can be prepared in good yield from a nucleoside in the presence of a high concentration of phosphate[304]. The isolated R-1-P is then used as the glycosyl donor in an enzymatic coupling reaction with added purine or pyrimidine bases or analogs. By this method, generally any heterocycle which is a substrate for a nucleoside phosphorylase can be glycosylated. The second strategy involves a one-pot exchange of one base for another in the presence of a catalytic amount of inorganic phosphate without isolation of R-1-P. At best, this procedure results in an equilibrium mixture of substrate and product nucleosides, from which the product must be isolated. In less favorable cases, the natural purine or pyrimidine base released from the glycosyl donor may be a potent competitive inhibitor versus the purine or pyrimidine analog. For example, competitive inhibition by hypoxanthine (K_M = 5.6 mM) was the cause for the lack of glycosylation of 1,2,4-triazole-3-carboxamide (TCA, the aglycon component of virazole, K_M = 167 mM) when inosine was used as the ribosyl donor and purine nucleoside phosphorylase (PNPase) as the catalyst[256]. It was, however, possible to synthesize virazole by isolating R-1-P and subsequently using it as the ribosyl donor[305]. An alternative way to circumvent the inhibition problem is to employ a pyrimidine nucleoside as the glycosyl donor and a purine (or purine analog) as the acceptor, since the released pyrimidine base does not inhibit the purine nucleoside phosphorylase[306]. By this method, both pyrimidine nucleoside phosphorylase and purine nucleoside phosphorylase are required. Direct purine-to-purine exchange reactions have been conducted without isolation of R-1-P using activated purine derivatives as the ribosyl donors[307].

The nucleoside phosphorylases accept a wide range of nucleoside analogs as

substrates, with modifications in both the base and glycosyl components. The use of unnatural bases has met with success using both natural and unnatural glycosyl donors. However, a few limitations have been observed, such as loss of appropriate regio-specificity with unnatural bases[306]. The synthesis of sugar-modified nucleosides has made use of glycosyl donors which are prepared by chemical modification of readily available nucleosides, such as uridine and cytidine. Good yields of arabino[303] and 2'-amino-2'-deoxyribonucleosides[309] have also been obtained enzymatically, although the enzymatic synthesis of 3'-amino-2',3'-dideoxyribonucleosides has given only low yields[310, 311]. The synthesis of ribosides of unnatural purine and pyrimidine bases and the synthesis of nucleosides containing modified glycosyl moieties are summarized in Table 11.3-8. Most of these reactions have been carried out in one step without isolation of the intermediate sugar phosphate, although involvement of the sugar phosphate intermediate has been demonstrated.

In summary, the nucleoside phosphorylases provide a regio- and stereo-specific route for nucleoside synthesis which is applicable to nucleoside analogs which are modified in either the base or the sugar moiety. These processes provide good yields of products in most cases without the extensive protection and deprotection steps involved in traditional chemical synthesis of nucleosides. Application of this strategy to the synthesis of 2'-deoxy- and 2',3'-dideoxynucleosides was reported with the use of *N*-deoxyribosyl transferase from *Lactobacillus* species[301, 312].

11.3.5.2
NAD Hydrolase

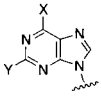
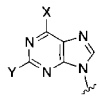
The enzyme NAD glycohydrolase has been used in exchange reactions for the preparation of NAD analogs[318]. The enzyme accepts nicotinamide analogs with modification at the amide functionality as substrates. Depending on the structure of the nicotinamide analogs used, the reaction may be either reversible or irreversible. NADH and its 6-hydroxyl derivative are not substrates for the enzyme. When 4-amino, 4-methylamino, or 4-dimethylamino nicotinamide or nicotinate was used as substrate, the product NAD analog existed as a 1,4-dihydro-type tautomer[319].

11.3.6
Biological Applications of Synthetic Glycoconjugates

11.3.6.1
Glycosidase and Glycosyl Transferase Inhibitors

Carbohydrate analogs and derivatives are valuable in studying the biosynthesis and modification of oligosaccharides: deoxynojirimycin, swainsonine, and castanospermine inhibit trimming of the *N*-linked oligosaccharides of glycoproteins[320]; tunicamycin and streptovirudin inhibit protein glycosylation in the Leloir pathway[321]; acarbose inhibits amylase[322]. These inhibitors provide a way of exploring cell-surface oligosaccharide chemistry, a topic of central interest in differentiation, development, and disease. Most are relatively easily understood as transition state

Table 11.3-8. Nucleoside phosphorylase-catalyzed synthesis with various heterocycles as acceptors or sugar-modified nucleosides as donors.

Donor	Acceptor		Method[a]	Yield (%)[b]	Ref.
Uridine		X = MeS, Y = H X = NH_2, Y = Cl	B	59–76	[313, 314]
Thymidine		X = Me_2N, Y = H	B	81	[313, 314]
7-*N*-Methyl Guanosine		X = NH_2, Y = H	B	100	[307]
Inosine		X = $C_5H_{11}S$, Y = H	A	59	[313]
Uridine		X = NH_2, BnNH	B	18–79	[313, 306]
Thymidine		X = Cl	B	18–71	[313, 306]
7-*N*-Methyl Guanosine		X = NH_2	B	53	[307]
Uridine		X = OH, PhCONH	B	23–63	[313, 315]
Inosine			A	47	[305]
7-*N*-Methyl Guanosine			B	44	[307]
 1-(β-D-arabinosyl)uracil		X = NH_2, Y = H, NH_2, CH_3 X = OH, Y = Cl, H, NH_2, CH_3 X = H, Y = NH_2 X = SH, Y = NH_2	B	34–92	[316]
 2'-amino-2'-deoxyuridine		X = NH_2, Y = H X = OH, Y = H, NH_2, Cl	B	20–50	[308, 309, 317]
 3'-amino-3'-deoxythymidine		X = F, Cl, Br	B	7–29	[310]
 R = H (5'-deoxyuridine) or R = OH (5'-deoxythymidine)			B	12–17	[306]

a Method A: α-Glycosyl-1-phosphate generated and isolated prior to addition of acceptor heterocycle.
Method B: *In situ* generation of α-glycosyl-1-phosphate.
b Yields are based on the initial amount of heterocycle acceptor.

analogs, and the design of new sugar analogs to inhibit other glycosidases and glycosyltransferases[182, 323] can be accomplished.

The syntheses of these types of structures are not straightforward using classical synthetic methods. Enzymatic methods have already been proven to be very useful in

syntheses of deoxynojirimycin and related materials [324], and are widely applicable to other similar structures.

11.3.6.2
Glycoprotein Remodeling

A number of the proteins employed as human pharmaceuticals (tissue plasminogen activator, juvenile human growth hormone, CD4, EPO) are glycoproteins. There is substantial interest in developing methods that will permit modification of oligosaccharide structures on these glycoproteins by removing and adding sugar units (*"remodeling"*) and in making new types of protein-oligosaccharide conjugates [325, 326]. Modification of the sugar components of naturally occurring or unnatural glycoproteins might increase serum lifetime, increase solubility, decrease antigenicity, and promote uptake by target cells and tissues.

Enzymes are plausible catalysts for manipulating the oligosaccharide content and

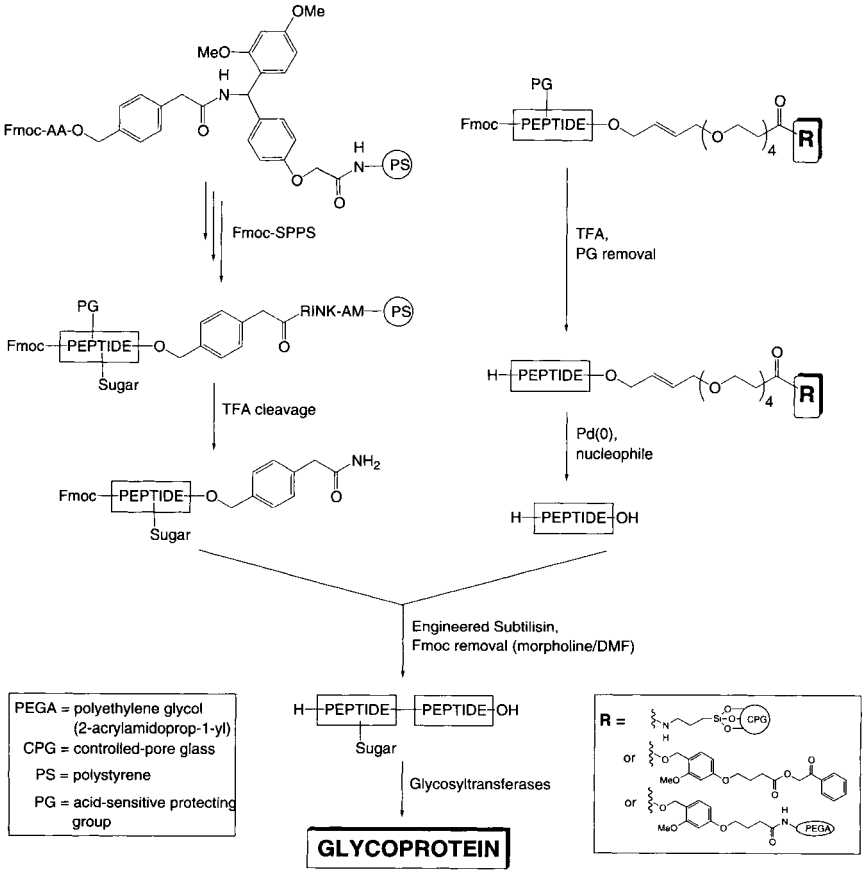


Figure 11.3-27.

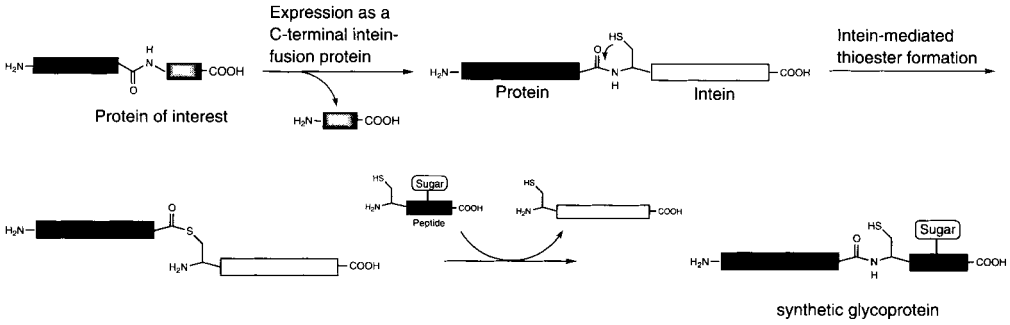


Figure 11.3-28.

structure of glycoproteins. The delicacy and polyfunctional character of proteins and the requirement for high selectivity in their modification indicate that classical synthetic methods will be of limited use. Major problems in enzymatic glycoprotein remodeling and generation are the unavailability of many of the glycosyltransferases and the uncertainty in glycosyltransferase specificity on the surface of novel proteins. Recent advances in this area have provided several new methods for the synthesis of homogeneous glycoproteins. Proteases have been utilized for glycopeptide bond ligation (Fig. 11.3-27) [327], specifically in the generation of a homogeneous RNase glycoform. Endo-glycosidases are capable of transforming heterogeneous glycans to homogeneous species in a single trans-glycosylation reaction [328]. Furthermore, intein-mediated splicing reactions allow modification of a protein C-terminus with carbohydrates or other molecular probes (Fig. 11.3-28) [329].

11.3.7
Future Opportunities

In general, the development of carbohydrate-derived pharmaceutical agents has occurred at a slower pace than that of other biomolecules, undoubtedly because of difficulties in their synthesis and analysis. However, distinct areas of biology and medicinal chemistry have directed attention at carbohydrates. Interfering with the assembly of bacterial cell walls [7, 330] remains one of the most successful strategies for the development of antimicrobials. As bacterial resistance to antibiotics of last resort (i.e. vancomycin) becomes more widespread, interest in developing new antipathogenic agents is increasing. Those based on carbohydrate components of the cell wall, such as KDO, heptulose, and Lipid A, represent novel targets. Cell-wall constituents are also relevant to vaccines and as leads toward non-protein immunomodulating compounds. Furthermore, cell-surface carbohydrates are central to differentiation and development, and may be relevant to abnormal states such as those characterizing some malignancies [7]. The broad interest in diagnostics has begun to generate interest in carbohydrates as markers of human health. In addition, there are a number of other possible applications of carbohydrates, for example as dietary constituents, in antivirals, or as components of liposomes for

drug delivery. Enzymatic methods of synthesis, by rendering carbohydrates more accessible, will contribute to further research in all of these areas.

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11.4
Natural Polysaccharide-degrading Enzymes

Constanzo Bertoldo and Garabed Antranikian

11.4.1
Introduction

Polymeric substrates such as starch, cellulose, hemicellulose and pectin are abundant in nature and provide a valuable and renewable source of carbon and energy. A diverse range of fungi, yeast, bacteria and archaea are capable of attacking such complex polymeric substrates by producing extracellular enzymes with a wide range of specificity. In this chapter we summarize the current state of knowledge on polysaccharide-degrading enzymes, and attempts are made to show their biotechnological significance.

11.4.2
Starch

Starch is the most economically important reserve polysaccharide in the plant kingdom and is in addition the major source of carbohydrates in human nutrition. In contrast to non-starch reserve polysaccharides, which are outside the cell and the plasmalemma, starch is located in the so-called plastids or in vacuoles within the plant cells [1]. In seeds, the highest starch content can be found in the endosperm, whereas its content in the embryo and the pericarp is very low. In general, the starch content of seeds or fruits varies with the degree of maturation [2]. Starch occurs in semicrystalline form in granules. The size and the shape of the granules is dependent on the plant species and may reach about 175 mm.

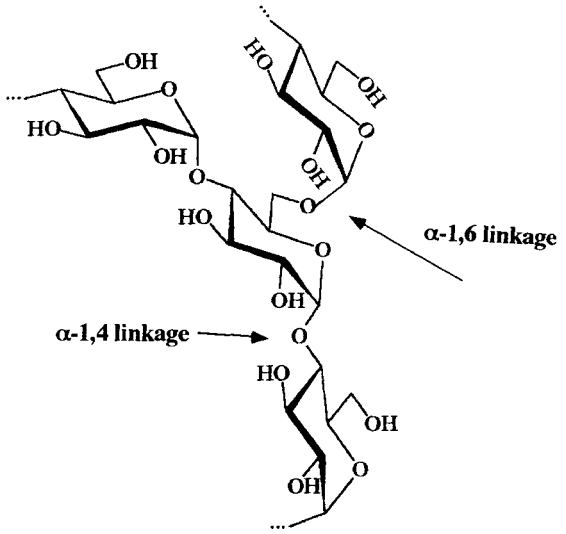


Figure 11.4-1. Structure of the branching point in amylopectin.

Starch is composed of amylose (15–25 %) and amylopectin (75–85 %). Amylose is a linear macromolecule consisting of 1,4-linked α-D-glucopyranose residues. The chain length varies from several hundred to 6000 residues[1]. The direction of the chain is characterized by the reducing and the non-reducing end. The reducing end is formed by a free C-1 hydroxyl group. Like amylose, amylopectin is composed of α-1,4-linked glucose molecules, but in addition branching points with α-1,6 linkages occur. The branching points occur at every 17–26 glucose molecules, so that the content of α-1,6 linkages in amylopectin is about 5 %[3]. With its molecular mass of 10^6 to 10^9, amylopectin is one of the largest biological molecules (Fig. 11.4-1).

The iodine-binding capacity of starch is dependent on the degree of polymerization (DP). Amylose forms with iodine a helical inclusion complex with an intense blue colour, which possesses an absorption maximum at wavelengths between 620 and 680 nm. Amylopectin has much less iodine-binding capacity because of its branched character, leading to a red-violet colour with absorption maximum of 540 nm[3].

11.4.2.1
Classification of Starch-degrading Enzymes

Starch-degrading enzymes can be divided into two classes according to their reaction mechanism: the glucosidases and the glycosyl-transferases. The first class, the glucosidases, are classified as hydrolases, which catalyze an irreversible hydrolytic cleavage of the glycosidic bond. The group of glucosidases is further subdivided, according their point of attack, into endoacting and exoacting enzymes. Endoacting enzymes hydrolyze linkages in a random manner in the inner part of the starch molecule, releasing linear and branched oligosaccharides with various chain length. α-Amylase is classified as an endoacting enzyme. In contrast to endoacting enzymes,

the exoacting enzymes hydrolyze linkages from the non-reducing end of the polysaccharide chain. This group includes β-amylase, glucoamylase and α-glucosidase. Isoamylase, pullulanases type I and pullulanase type II are classified as debranching enzymes.

Glycosyltransferases transfer glycosyl groups from a starch chain to an acceptor. The acceptor may be another starch molecule, phosphoric acid or nucleotides. Most enzymes in this class catalyze reversible reactions; some enzymes are involved in the starch biosynthesis. The only glycosyltransferase responsible for starch degradation is the cyclodextrin glycosyltransferase.

11.4.2.2
α-Amylase (1,4-α-D-Glucan,4-Glucanhydrolase, E.C. 3.2.1.1)

Amylases are widely distributed in plants, mammalian tissues and microorganisms. The endoacting enzymes produce oligosaccharides and glucose as end products by hydrolyzing the α-1,4-glycosidic linkages in a random manner. The enzyme catalyzes multichain attack as well as multiple attack on the same chain[4]. Amylose is hydrolyzed to maltose and glucose. The anomeric carbon in all products formed has the α-D configuration. α-Amylase is not able to attack α-1,6 linkages in amylopectin and glycogen. The α-1,4 linkages in the vicinity of branching points are also not attacked by this enzyme[5]. In spite of this, the enzyme is capable of bypassing the branching points. Therefore, the action of α-amylase on branched substrates results in the formation of α-limit dextrins. The structure of the α-limit dextrin is dependent on the source of α-amylase. α-Amylases are also described as liquefying and saccharifying enzymes. The saccharifying α-amylases reduce the viscosity less than liquefying enzymes and attack the substrate repetitively[6]. Most enzymes have an absolute requirement for calcium ions, and the temperature optima as well as the temperature stability of α-amylases are significantly enhanced in the presence of calcum ions and substrate. α-Amylases are widely distributed among microorganisms, including aerobic and anaerobic bacteria and archaea as well as actinomyces, fungi and yeasts. α-Amylases are produced by a variety of *Bacillus* species, like *B. amyloliquefaciens*, *B. cereus*, *B. circulans*, or *B. subtilis*[7–10]. The *Bacillus* enzymes are characterized by a wide range of temperature and pH optima[1]. The α-amylase from *B. acidocaldarius* shows optimal activity at pH 3.5 and 75 °C; the enzyme from *Bacillus* sp NRRL B2881 prefers alkaline conditions (pH optimum at pH 9.2) and 50 °C[11, 12]. Anaerobic microorganisms belonging to the genera *Clostridium*, *Thermoanaerobacter*, *Thermoanaerobium* and *Thermobacteroides* have also been reported to synthesize extracellular, amylolytic enzymes[13]. Also the α-amylases from archaea have been characterized (e.g.: *Pyrococcus furiosus*, *Thermococcus profundus*). Some of these enzymes are optimally active above 100 °C[14, 15].

11.4.2.3
β-Amylase (1,4-α-D-Glucan Maltohydrolase, E.C. 3.2.1.2)

β-Amylases occur in most higher plants and a number of microorganisms, and are absent in mammalian tissues. The exoacting β-amylases hydrolyze α-1,4 linkages by the stepwise removal of maltosyl residues from the non-reducing end of polysaccharides[16]. During the hydrolysis an inversion of the anomeric configuration occurs leading to β-maltose as end product. Unlike α-amylase, β-amylase cannot bypass the α-1,6 linkages in branched substrates and stops two or three glucose units before the branching point. In amylopectin or glycogen, hydrolysis occurs only in the outer chains, and therefore maltose and α-limit dextrins of high molecular weight are the endproducts. β-Amylase is produced by few *Bacillus* species. The pH optima determined for the *B. megaterium* and the *B. polymyxa* enzymes are in the neutral and slightly alkaline region and the enzymes are unstable above 60 °C. The β-amylase from *Clostridium thermosulfurogenes* ATCC 33743 was characterized as a thermoactive enzyme with a temperature optimum of 75 °C[17, 18]. So far, this is the only β-amylase produced by an anaerobic microorganism.

11.4.2.4
Glucoamylases (1,4-α-D-glucan glucohydrolase, E.C. 3.2.1.3)

Glucoamylases, also termed amyloglucosidase or γ-amylase, are produced predominantly by fungi, especially by species of *Aspergillus*, *Rhizopus* and *Endomyces*. They are rare in procaryots and absent in plants or in mammalian tissues.

The enzyme acts similarly to β-amylase, but attacks α-1,4 as well as α-1,6 linkages from the non-reducing end. β-D-glucose is released as an end product. Glucoamylases are not specific for α-1,4 and α-1,6 linkages; hydrolysis of α-1,3 linkages has also been reported[19]. The enzyme prefers polysaccharides for rapid hydrolysis and has lower affinity to oligosaccharides or maltose. Because of the lower affinity to α-1,6 linkages, the rate of starch hydrolysis decreases subsequentially. In practice, a complete degradation of amylopectin or branched substrates could not be observed[20]. Pullulan hydrolysis from the non-reducing end by glucoamylases to glucose was also reported[21, 22]. (For a description of pullulan see 1.2.1.5.). The glucoamylases produced by *Aspergillus* species and yeast are active in the acidic range (pH 4–5 for the fungi and pH 2.5–5.5 for the yeast). The enzymes are unstable above 60 °C. The presence of glucoamylase in the thermophilic anaerobic bacterium *Clostridium thermosaccharolyticum* was reported by Specka et al. (1992) and very recently in the thermoacidophilic archaea *Picrophilus oshima*, *Picrophilus torridus* and *Thermoplasma acidophilum*[14]. These enzymes are optimally active at 90 °C and pH 2.0.

11.4.2.5
α-Glucosidase (α-δ-Glucoside Glucohydrolase, E.C. 3.2.1.20)

α-Glucosidase catalyzes the hydrolysis of terminal α-1,4 linkages from the non-reducing end in different substrates. The product released is α-D-glucose. The enzyme prefers short-chain oligosaccharides as substrates and has very low affinity to polysaccharides. In addition, many α-glucosidases show activity towards maltose, acylglucoside, alkylglucoside and isomaltose[24]. α-Glucosidases are produced by many *Bacillus* and *Aspergillus* strains and may be present in several industrial enzyme preparations as side activities.

Intracellular α-glucosidases are produced by many microorganisms and are also widely distributed among animals and plants. α-Glucosidases formed by various *Bacillus* species (*B. subtilis, B. amyloliquefaciens, B. cereus*), *Pseudomonas* (*P. amyloderamosa, P. fluorescens* W) and lactic acid bacteria (*Lactobacillus acidophilus, Streptococcus pyogenes*) are active at slight acidic pH at temperatures up to 75 °C. (for review see[24–26]). α-Glucosidases produced from thermophilic *Clostridia* and archaea are extremely thermostable and thermoactive. The highest activity for clostridial and archaeal enzymes is determined from 65 to 90 °C, and from 105 to 115 °C, respectively. α-Glucosidases vary in their substrate specificity. In addition to α-1,4-hydrolyzing activity, some enzymes show low α-1,6-hydrolyzing activity and are capable of hydrolyzing isomaltose. Interestingly, the α-glucosidase from *B. thermoglucosidasius* KP 1006 is unable to hydrolyze maltose but attacks isomaltose with high affinity[27]. The enzymes from *B. cereus* and from *P. amyloderamosa* are reported to hydrolyze besides α-1,4 and α-1,6 linkages also α-1,2 and α-1,3 glucosidic bonds[28].

11.4.2.6
Isoamylase (Glycogen 6-Glucanohydrolase, E.C. 3.2.1.68)

Isoamylase hydrolyzes with high specificity the α-1,6 linkages in branched substrates such as amylopectin or glycogen. The enzyme cannot catalyze a complete degradation of α- or β-limit dextrins, although the smallest subtrate is ^{2}6-α-D-malto triosylmaltose[2]. Branched substrates are completely debranched, but isoamylase is unable to attack pullulan. Pullulan is an α-D-glucan synthesized by the yeast *Aureobasidium pullulans* and consists of about 480 maltotriose units linked by α-1,6-D bonds (Fig. 11.4-2). Pullulan is used as a model substrate for starch debranching enzymes, because the α-1,6 linkages seem to imitate to a certain degree the α-1,6-branching points in substrates like amylopectin. Isoamylase has higher affinity to large branched polysaccharides. The enzyme is very rare among microorganisms and has been detected in *Pseudomonas amyloderamosa* and *Cytophaga* sp.[29, 30].

11.4.2.7
Pullulanase Type I (α-Dextrin 6-Glucanohydrolase, E.C. 3.2.1.41)

Pullulanase type I hydrolyzes α-1,6 linkages in amylopectin, pullulan or limit dextrins with high specificity. Pullulan is completely degraded in a random fashion

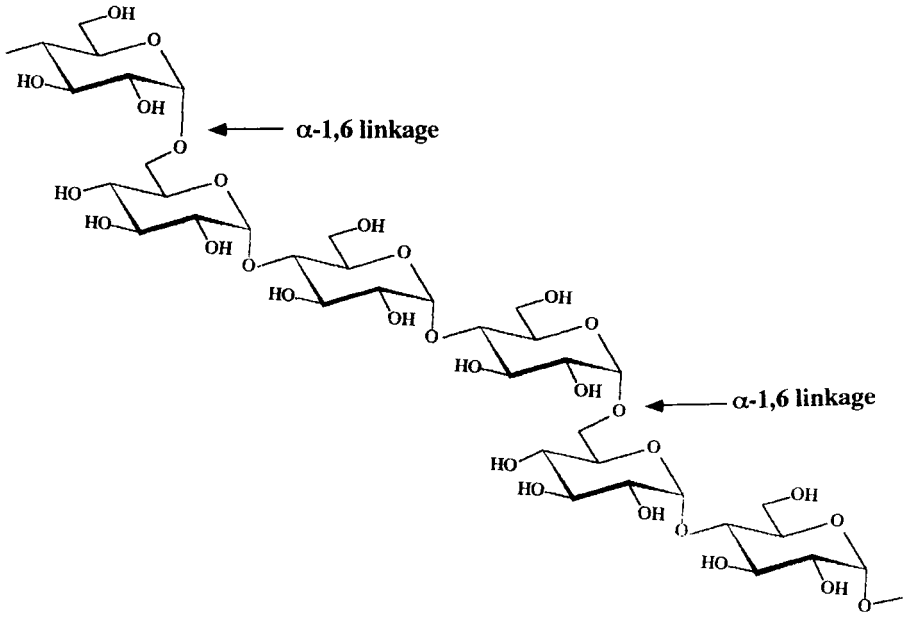


Figure 11.4-2. Structure of pullulan.

to maltotriose, whereas native glycogen is not attacked by the enzyme. Substrates with short branches such as β-limit dextrin are hydrolyzed at a higher rate than amylopectin[31]. Pullulanase I requires at least two α-1,4 linked glucose units in the vicinity of the α-1,6 linkage[32]. The smallest substrate for pullulanse I was reported by Marshall to be ^{2}6-α-D-maltosylmaltose[21]. Pullulanase I catalyzes a condensation reaction in the presence of maltotriose or maltose at high enzyme concentration; the condensation products contain α-1,6 linkages[33]. Pullulanases type I are predominantly formed by mesophilic microorganisms such as *Klebsiella pneumoniae, Bacillus acidopullulyticus, B. cereus* var mycoides, *B. macerans* ' *B. polymyxa* and *Streptomyces mitis*[2]. Fervidobacterium pennivorans is one of the few anaerobic bacteria which produce heat-stable pullulanase type I[14].

11.4.2.8
Pullulanase Type II or Amylopullulanase

Unlike pullulanase I, pullulanase II hydrolyzes α-1,6 linkages in pullulan and in addition is capable to cleave α-1,4 linkages in amylose[34, 35]. These enzymes with dual specificity belong to a new class of pullulanases, termed pullulanase type II or amylopullulanase[13, 36, 37]. Pullulanase I and II are absolutely unable to hydrolyze substrates like dextran or isomaltotriose, which contain exclusively α-1,6 linkages. They possess an absolute requirement for α-1,4 linkages in the vicinity of the α-1,6 linkages[13, 26]. Pullulanase type II is widely distributed in anaerobic microorganisms including species of the genera *Clostridium, Dictyoglomus, Thermoanaerobacter, Thermoanaerobium, Thermobacteroides* and *Pyrococcus*. These enzymes are extremely

thermostable and are optimally active in the temperature range between 75 and 105 °C[14, 38].

11.4.2.9
Pullulan Hydrolases (Type I, Neopullulanase; Type II, Isopullulanase, E.C. 3.2.1.57, Pullulan Hydrolase Type III)

Pullulan hydrolase type I (neopullulanase) and pullulan hydrolase type II (isopullulanase) hydrolyze the α-1,4 linkages in pullulan, liberating panose and isopanose, respectively. Both enzymes are unable to hydrolyze α-1,6 glycosidic bonds in branched substrates or pullulan. Due to this fact, the classification of pullulan hydrolases into the group of debranching enzymes is misleading. Pullulan hydrolases type I have been described from *B. stearothermophilus* and *B. stearothermophilus* KP 1064 and pullulan hydrolase type II from *Arthrobacter globiformis* T6[39, 58]. Recently, pullulan-hydrolase type III from *T. aggregans* has been detected cloned and expressed in mesophilic hosts. This enzyme attacks α-1,4 as well as α-1,6 glycosidic linkages in pullulan, producing maltotriose, maltose, panose and glucose[58].

11.4.2.10
Cyclodextrin Glycolsyltransferase (1,4-α-D-Glucan 4-α-D-(1,4-α-D-Glucano)-Transferase, E.C. 2.4.1.19)

Cyclodextrin glycosyltransferases are produced predominantly by *Bacillus* species (*B.circulans, B. stearothermophilus, B. macerans, B. megaterium*), *Klebsiella pneumoniae* and *Micrococcus* sp. (for review see[40]). The extracellular enzymes produced by *Bacillus macerans* and *B. megaterium* catalyze the transformation of linear chains of starch into cyclic oligosaccharides, the Schardinger cyclodextrins. The glucose residues in cyclodextrins are linked by α-1,4-glycosidic bonds and, because of the ring structure, reducing ends are absent. α-, β- and γ-cyclodextrins consist of 6, 7 and 8 glucose units, respectively. The specificity, the source and the type of the enzyme are responsible for the ratio of different cyclodextrins formed. In principle, all cyclodextrin glycosyltransferases produce α-, β- and γ-cyclodextrins simultaneously[56, 57]. Thermostable cyclodextrin glycosyltransferases (CGTases) are produced by *Thermoanaerobacter* species, *Thermoanaerobacterium thermosulfurigenes* and *Anaerobranca gottschalkii*. Recently, a CGTase, with optimal temperature at 100 °C, was purified from a newly isolated Archaeon, *Thermococcus* sp. This is the first report of the presence of a thermostable CGTase in a hyperthermophilic Archaeon[14].

The occurrence of different starch-degrading enzymes in microbes is summarized in Table 11.4-1.

11.4.2.11
Biotechnological Applications of Starch-degrading Enzymes

Starch-degrading enzymes are applied in the starch bioprocessing, sugar, alcohol and brewing industries. The commercially most important application of starch-

Table 11.4-1. Occurrence of different starch hydrolyzing enzymes in microorganisms.

Enzymes	Substrate (enzyme action)	Products	Organisms
α-Amylase	starch (endoacting α-1,4)	α-limit dextrins branched oiligo-saccharides, glucose maltose, linear oligomers	*Bacillus amyloliquefaciens* *B. cereus* *Thermotoga maritima* *Pyrococcus furiosus*
β-Amylase	starch (exoacting α-1,4)	β-maltose limit dextrins	*Bacillus megaterium* *B. polymyxya* *Clostridium thermosulfurogenes*
Glucoamylase	polysaccharides [exoacting α-1,4,(α-1,6)]	β-D-glucose	*Aspergillus niger, A. oryzae* *Rhizopus nodosus* *Clostridium acetobutylicum* *Picrophilus torridus*
α-Glucosidase	oligosaccharides [exoacting α-1,4,(α-1,6)]	α-D-glucose	*Bacillus subtilis* *B. cereus* *Streptococcus pyogenes* *Thermococcus strain ANI* *T. hydrothermalis*
Isoamylase	branched polysaccharides [endoacting α-1,6]	linear polysaccharides	*Pseudomonas amyloderamosa* *Flavobacterium odorratum*
Pullulanase Type I	pullulan, branched polysaccharides [endoacting α-1,6]	maltotriose, linear oligosaccharides	*Klebsiella pneumoniae* *Bacillus acydopullulyticus* *Fervidobacterium pennivorans* *Thermotoga maritima*
Pullulanase Type II	pullulan, branched polysaccharides [endoacting; α-1,6 in pullulan; α-1,6 + α-1,4 in branched poly- and oligosaccharide]	maltotriose, linear oligosaccharides	*B. subtilis* *C. thermohydrosulfuricum* *Pyrococcus woesei* *Desulfurococcus mucosus*
Pullulan-hydrolase Type I	pullulan [α-1,4]	panose	*B. stearothermophilus*
Pullulan-hydrolase Type II	pullulan [α-1,4]	isopanose	*Arthrobacter globiformis*
Pullulan-hydrolase Type III	pullulan [α-1,4]	glucose, maltose maltotriose	*Thermococcus aggregans*
Cyclodextrin-glycosyl-transferase	branched polysaccharides [endoacting α-1,4]	α-β-γ-cyclodextrin	*B. circulans* *B. macerans* *Anaerobranca gottschalkii* *Thermococcus sp.*

* Values in brackets () indicate low enzyme affinity

degrading enzymes is the production of syrups and sweeteners. The conversion of corn starch to fructose begins with a liquefaction step carried out with α-amylase from *B. licheniformis* at 105–115 °C and 90–95 °C at pH 6. Amylose and amylopectin are hydrolyzed to dextrins and some oligosaccharides. The saccharification follows the liquefaction in the presence of glucoamylase from *Aspergillus niger* and debranching enzymes. The process conditions in the saccharification step have to be changed since the enzymes are optimally active at pH 4–4.5 and 55–65 °C. A dextrose solution of 95% results from this step. The dextrose solution can be crystallized or subsequently further isomerized. The isomerization from glucose to fructose again requires variation of the process conditions (55–60 °C, pH 7–8)[41, 42]. The finding of different amylolytic enzymes that are active under the same conditions will certainly improve the starch bioconversion process. Recently, it was found that hyperthermophilic microorganisms are a good source of such enzymes. α-Amylase, pullulanase and α-glucosidase from *Pyrococcus* sp. are optimally active at pH 4–5 and 100–110 °C[26].

In the baking industry α-amylase from fungi is used in order to release dextrin and fermentable sugars for yeast metabolism. Exhaustive dextrin formation, however, will lead to undesirable properties like loaf stickness and dark color.

In the process of fuel alcohol production different grains and tubers serve as raw material in the fermentation process. The liquefaction and saccharification steps are carried out in the presence of α-amylase and glucoamylase, respectively. This saccharified feedstock forms the substrate for the ethanol fermentation with yeasts[41]. Pullulanases are also used in the production of "light beer", which has low carbohydrate content. During the fermentation process the pullulanase is added together with fungal α-amylase or glucoamylase to the wort[38]. The production of branched and more water-soluble cyclodextrins can also be carried out with pullulanase. The pullulanase catalyzes the transfer reaction of malto-oligosaccharides to cyclodextrins. Branched cyclodextrins are more water-soluble than linear cyclodextrins[43]. CGTases are used for the production of cyclodextrins that can be used as a gelling, thickening or stabilizing agent in jelly desserts, dressing, confectionery, dairy and meat products. Because of the ability of cyclodextrins to form inclusion complexes with a variety of organic molecules, cyclodextrins improve the solubility of hydrophobic compounds in aqueous solution. This is of interest for the pharmaceutical and cosmetic industries. Cyclodextrin production is a multistage process in which starch is first liquefied by a heat-stable amylase, and in the second step a less thermostable CGTase from *Bacillus* sp. is used. The application of heat-stable CGTase in jet cooking, where temperatures up to 105 °C are achieved, will allow liquefaction and cyclization to take place in one step.

11.4.3
Cellulose

Cellulose is the principal component of plant cell walls, and thus represents the world's most abundant organic polymer, with an annual production of 4×10^{10} tonnes per year. Cellulose is found in nature as an unbranched insoluble polymer

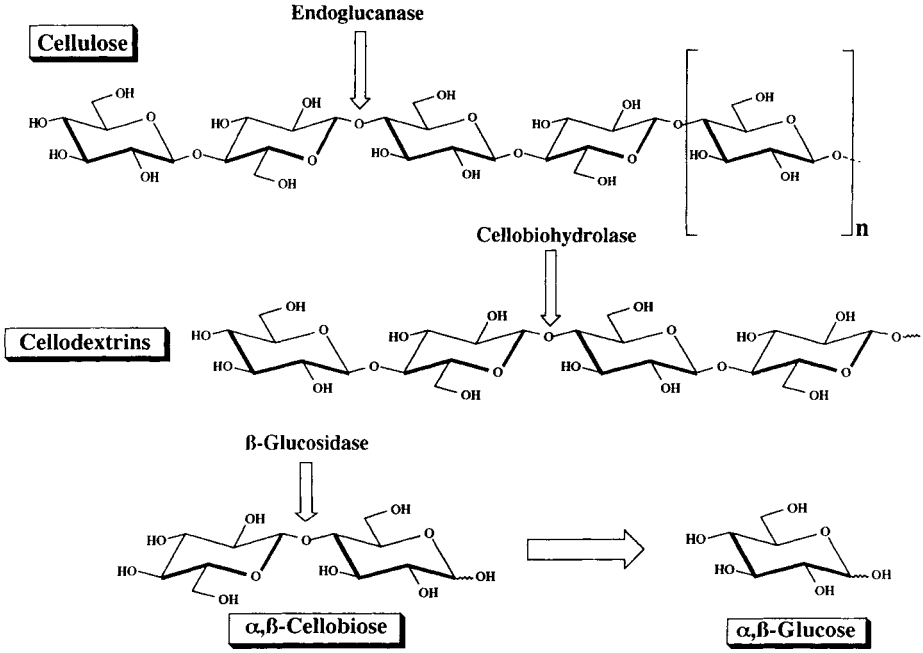


Figure 11.4-3. Action of cellulolytic enzymes on cellulose.

(Fig. 11.4-3) containing up to 14000 glucose units linked together by β-1,4-D-glycosidic bonds[59, 1]. The hydrogen capacity between individual chains in cellulose is quite high, with each residue contributing up to three OH groups. The individual chains of cellulose tend to form microfibrils as a result of inter- and intramolecular hydrogen bonding. The microfibrils associate in a similar way to form fibers[1,60, 61]. Cellulose contains both crystalline and amorphous regions. The term "crystalline" refers to those regions in which a high degree of order is found within and between the fibrils. In the amorphous regions, however, a lesser degree of order is predominant[62, 63]. Crystalline regions are more resistant to degradation than amorphous regions[64, 65]. Thus, the fraction of crystalline regions found in cellulose is an important factor affecting the rate and enzymatic hydrolysis of cellulose[61, 66]. Cellulose is found in nature as a principal structural element in cell walls of higher plants, in association with hemicellulose, lignin and other polysaccharides[67]. Cellulose is also found in some seaweeds and can be synthesized by some bacteria[68]. Cellulose occurs in an almost pure form (98%) in cotton fibers, while in flax (80%), jute (60–70%), wood (40–50%), and forages (24–36%) a less pure form of cellulose is found[1, 62, 69].

11.4.3.1
Cellulose-degrading Enzyme Systems

Cellulases are a group of enzymes capable of hydrolyzing insoluble cellulose to its monomer glucose[70]. Because of the crystalline and insoluble nature of cellulose, its degradation is very slow. Cellulose degradation requires a multienzyme complex involving at least three major enzymes, namely 1,4-β-D-glucan glucanohydrolase (endoglucanase, E.C. 3.2.1.4), 1,4-β-D-glucan cellobiohydrolase (exoglucanase, E.C. 3.2.1.91), and β-D-glucoside glucohydrolase (β-glucosidase, E.C. 3.2.1.21)[71] (Fig. 11.4-3). Mainly two types of enzyme systems have been recognized to be involved in cellulose hydrolysis. The first is the non-aggregating system, in which the three main cellulolytic enzymes are produced and mainly found to be secreted into the growth medium as separate entities. In this system, endoglucanase, cellobiohydrolase and β-glucosidase act in synergy[129, 72]. The second enzyme system is referred to as the aggregating system. This system is found mainly in anaerobic bacteria, where cellulases are secreted as a high molecular weight multienzyme complex. This complex is generally found on the cell surface, where it mediates the attachment between the cells and the substrate[129]. The most studied system is that of *Clostridium thermocellum*[73]; the complex is named "cellulosome".

11.4.3.2
Endoglucanase (1,4-β-D-Glucan-Glucanohydrolase, E.C. 3.2.1.4)

Endoglucanase hydrolyzes cellulose randomly, producing oligosaccharides, cellobiose and glucose as end products (Fig. 11.4-3). Endoglucanase attacks mainly the amorphous regions in cellulose and soluble derivatives of cellulose[74]. The action of endoglucanase results in a decrease in the chain length of carboxymethylcellulose (CMC), acid-swollen cellulose and soluble barley glucan, producing mainly glucose, cellobiose, cellotriose and other oligosaccharides[64, 75, 76]. Substrates like *p*-nitrophenyl-β-D-cellobioside and methylumbelliferyl-β-D-cellobioside are hardly attacked by endoglucanase. Low activity is also observed with microcrystalline cellulose[77]. In contrast to this, the endoglucanase of *Trichoderma viride* shows high activity towards crystalline cellulose but only weak activity towards CMC[80].

11.4.3.3
Cellobiohydrolase (1,4-β-D-Glucan Cellobiohydrolase, E.C. 3.2.1.91)

Cellobiohydrolases are exoglucanases that attack the non-reducing end of the cellulose polymer chain to produce cellobiose (Fig. 11.4-3). Recent reports have indicated that the attack on cellulose is not restricted to the end of the chain. Thus, the cellobiohydrolase I from *Trichoderma reesei* is capable of degrading the β-glucan from barley in a manner typical of an endoglucanase[81]. Cellobiohydrolases comprise the major part of fungal cellulase systems that are capable of degrading crystalline cellulose[72]. Up to 80% of microcrystalline cellulose can be degraded by this enzyme[71]. Earlier studies have indicated the absence of cellobiohydrolases in

bacterial cellulase systems[75]. However, Langsford et al.[82] reported the presence of this enzyme in *Cellulomonas fimi*. This enzyme has also been found in *Ruminococcus albus*[83] and *R. flavefaciens*[84]. Bacterial cellobiohydrolases are capable of hydrolyzing model substrates such as *p*-nitrophenyl-β-D-cellobioside and methylumbelliferyl-β-D-cellobioside. They release cellobiose from microcrystalline cellulose and show low activity towards CMC[77].

11.4.3.4
β-Glucosidase (β-d-Glucoside Glucohydrolase, E.C. 3.2.1.21)

Cellobiase or β-glucosidase acts mainly on cellobiose and cellodextrins (up to DP of 6) to produce β-glucose (Fig. 11.4-3); cellulose and higher cellodextrins are not hydrolyzed by this enzyme[64]. β-Glucosidase acts also on sophorose and cellobiose to produce monosaccharides. In addition, model substrates such as *p*-nitrophenyl-β-D-glucosides or methylumbelliferyl-β-D-glucoside are attacked. Because of the action of β-glucosidase, the inhibitory effect of cellobiose on cellobiohydrolase and endoglucanase can be removed. As shown for the β-glucosidase from *Penicillium funiculosum*, this enzyme also acts synergistically with endoglucanases and cellobiohydrolases[85].

11.4.3.5
Fungal and Bacterial Cellulases

Most of the studies on cellulases have been conducted using fungal cellulolytic systems. Relatively few cell-free cellulases have been reported to degrade crystalline cellulose. Such fungal systems contain extracellular endoglucanase and cellobiohydrolase activities that convert crystalline cellulose to cellobiose[86]. The conversion of cellobiose to β-glucose is catalyzed by β-glucosidase, which has been found in the cultures of *Trichoderma viride*[87], *T. reesei*[88], *T. koningii*[89] and *Talaromyces emersonnii*[90] (Table 11.4-2). Compared to fungal systems, cell-free supernatants from cultures of cellulolytic bacteria seems to lack activity against crystalline cellulose[86]. Several cellulolytic bacteria have been isolated, but their cellulases have not been fully characterized[91, 92]. The system from *Cellulomonas* sp. is one of the most studied cellulolytic systems in bacteria[93, 94]. Many species which belong to the genera *Bacillus, Pseudomonas, Streptomyces, Thermoactinomyces* and *Thermomonospora* are capable of producing cellulolytic enzymes[91]. Several endoglucanases were detected in the culture fluid of many of these microorganisms. However, cellobiohydrolase has not been detected[86]. The hydrolysis of cellulose by bacteria involves the action of cellulolytic enzyme complexes consisting of different multicomponents. These complexes are associated with the cell wall of the bacterium and are often tightly bound to the cellulosic substrate[70]. They are released into the culture fluid only after extensive hydrolysis of cellulose. The most thoroughly studied cellulolytic enzyme complex, referred to as "cellulosome", is that of *Clostridium thermocellum*[73, 95].

Table 11.4-2. Microbial cellulolytic enzymes.

Organism	Endoglucanase	Cellobiohydrolase	β-Glucosidase
Fungi			
Aspergillus niger	+	–	+
Humicola insolens	+	–	+
Tricoderma koningii	+	+	+
T. reesei	+	+	+
T. viride	+	+	+
Bacteria:			
Cellulomonas fimi	+	+	–
Clostridium thermocellum	+	–	+
C. stercorarium	+	+	–
Cytophaga sp.	+	+	–
Fibrobacter succinogenes	+	–	+
Ruminococcus albus	+	+	+
Thermotoga maritima	+	+	–
Thermotoga neapolitana	+	+	–
Archaea			
Pyroccus furiosus	–	+	+
Sulfolobus solfataricus	–	–	+

11.4.3.6
Structure and Synergistic Effect of Cellulases

11.4.3.6.1 The "Cellulosome" Concept

The cellulolytic enzyme sytem of bacteria forms aggregates, which are associated with the cell wall forming catalytically active "protuberances". Electron microscopy studies revealed that these "protuberances" are found on the surface of all cellulolytic bacteria studied, whereas they are absent on the surfaces of non-cellulolytic bacteria[73, 96, 97]. In addition, they are not present during growth in the absence of cellulose[96]. The best characterized aggregation system is the cellulosome of *Clostridium thermocellum*[92, 95, 73]. The cellulosome binds to the substrate and is active towards crystalline cellulose. A 200 kDa polypeptide seems to be responsible for the substrate binding. In the early stages of growth the cellulosomes of *C. thermocellum* form polycellulosomes, which appears as protuberances on the cell surface. In the late growth phase the cellulosomes are released into the culture supernatant[77]. Cellulases present in the culture fluid seem to represent only fragments of cellulosomes and polycellulosomes[70]. Other cellulolytic bacteria which express cellulosome-like structures are *Ruminococcus albus, R. flavefaciens, Fibrobacter succinogenes*[96, 98], *Acetivibrio cellulolyticus, Bacteroides cellulosolvens, Clostridium cellobioparum, C. cellulovorans* and *Cellulomonas* sp.[96, 99]. Cellulose-degrading enzymes from various thermophilic organisms (*Thermotoga maritima, Thermotoga neapolitana, Caldocellum saccharolyticum* and *Anaerocellum thermophilum*) have been cloned, purified, and characterized. Recently, a thermostable archaeal endoglu-

canase which is capable of degrading β-1,4 bonds of β-glucans and cellulose has been characterized from *Pyrococcus furiosus*[14].

11.4.3.6.2 Multiple Forms of Cellulases

The cellulolytic enzymes from bacteria and fungi (endoglucanase, cellobiohydrolase and β-glucosidase) exist in multiple forms. Multiple forms of these enzymes seem to arise through post-translational modification by physiologically regulated processing activity or through post-secretional modification by proteolytic digestion[71]. Diversity of endoglucanases and cellobiohydrolases have been reported by several investigators[64, 100, 101]. However, *Penicillium notatum* and *Stereum sanguindentum* produce a single cellulase and are still able to degrade cellulose[102, 103]. Wilson[104] isolated five different endoglucanases from a protease negative mutant of *Thermomonospora fusca*; cellobiohydrolase activity, however, was not detected. Similarly, Shoemaker and Brown[105] identified four endoglucanases from *Trichoderma viride*. Further studies with *T. viride* proved the presence of six endoglucanases (Endo I, II, III, IV, V and VI), three cellobiohydrolases (Exo I, II and III) and one β-glucosidase[80].

11.4.3.6.3 Synergism

It has been recognized that the rate of hydrolysis of crystalline cellulose by the combination of endoglucanase and cellobiohydrolase is much faster than the sum of the individual actions of the components[70]. The rationale for the synergy of cellulase has been postulated as follows. The attack is initiated by a randomly-acting endoglucanase in the amorphous areas of the cellulose creating numerous new nonreducing ends that are attacked by cellobiohydrolase, resulting in the release of cellobiose. The β-glucosidase is needed for the removal of cellobiose, a strong inhibitor of both endoglucanase and cellobiohydrolase[72, 106]. Studies with the cellulases from *T. reesei* and bacteria showed that cellobiohydrolase II was only able to attack one end of the microcrystalline cellulose. However, in the presence of their endoglucanase, several sites of cellobiohydrolase attack at the amorphous region were observed[107]. Another observation showing the synergism between endoglucanase and cellobiohydrolase has been reported with the fungus *Neocallimastix frontalis*. Heat inactivation of the cellulases of this fungus resulted in loss of its ability to degrade crystalline cellulose. Interestingly, the endoglucanase activity was still measurable. The additon of cellobiohydrolase from *Trichoderma koningii* restablished the ability of the system to degrade cotton fibre[72]. Synergism between β-glucosidase and cellobiohydrolase or endoglucanase has also been observed. β-Glucosidase produced by *T. koningii* has shown synergism with cellobiohydrolase but not with endoglucanase[108]. Synergism between β-glucosidase and cellobiohydrolase can be explained by the ability of β-glucosidase to hydrolyze cellobiose, a strong inhibitor of cellobiohydrolase[109].

11.4.3.6.4 Biotechnological Applications of Cellulases

Cellulase preparations have found different biotechnological applications in several industrial processes. The most effective commercial cellulase is the one produced by *Trichoderma* species. Other cellulases of commercial interest are obtained from strains of *Aspergillus*, *Penicillium* and *Basidomycetes*. Fungal cellulases have been recommended for use in alcohol production. The alcohol yield from cassava is significantly increased if cellulases from *Trichoderma* sp. are added[110]. Cellulolytic enzymes can also be used to improve juice yields and effective color extractions of juices. Cellulolytic enzymes also improve the silage-making process[130]. The cellulase from *Trichoderma reesei* has been reported to accelerate the rate of ensilage processing when treating grass, lucerne and red clover[111]. The presence of cellulases in detergents causes colour brightening, softening and improved particulate soil removal[112]. A novel application of cellulases in textil industry is the use of Denimax (Novo Nordisk) for the "biostoning" of jeans instead of the classical stones in stone-washed jeans[113]. Another application of cellulases includes the pretreatment of cellulosic biomass and forage crops to improve nutritional quality and digestibility, enzymatic saccharification of agricultural and industrial wastes and production of fine chemicals[130].

11.4.4 Xylan

Hemicelluloses are non-cellulosic low molecular weight polysaccharides that are found together with cellulose in plant tissues[131]. In the cell walls of land plants, xylan is the most common hemicellulosic polysaccharide, representing more than 30% of the dry weight[132]. Most xylans are heteropolysaccharides which are composed of 1,4-linked β-D-xylopyranosyl residues[133, 134, 135]. This backbone chain is substituted with acetyl, arabinosyl, and glucuronosyl residues[133]. Homoxylans, on the other hand, consist of xylosyl residues exclusively and have been isolated from esparto grass[136], tobacco stalks[137], and guar seed husk[138].

The xylan of hardwoods (*O*-acetyl-4-*O*-methylglucuronoxylan) consists of at least 70 β-xylopyranose residues (average degree of polymerization between 150 and 200) linked by β-1,4-glycosidic bonds (Fig. 11.4-4)[139]. Every tenth xylose residue carries a 4-*O*-methylglucuronic acid attached to the C-2 of xylose[131]. In addition, hardwood xylans are highly acetylated; e.g. birchwood xylan contains more than 1 mol of acetic acid per 2 mols of xylose[140]. Acetylation occurs usually at the C-3 rather than the C-2 position of xylose. Acetylation at both positions has also been reported[141, 142]. The presence of these acetyl groups is responsible for the partial solubility of xylan in water[133]. The alkali extraction of xylan leads to the deacetylation of this substrate[140].

Softwood xylans (arabino-4-*O*-methyl-glucuronoxylans) are composed of shorter chains with a degree of polymarization between 70 and 130 (Fig. 11.4-5). Unlike hardwood xylan, the softwood xylan has a higher content of 4-*O*-methyl-glucuronic acid. The acetyl groups are replaced by α-L-arabinofuranose units, which are linked by α-1,3-glycosidic bonds to the C-3 position of xylose[143].

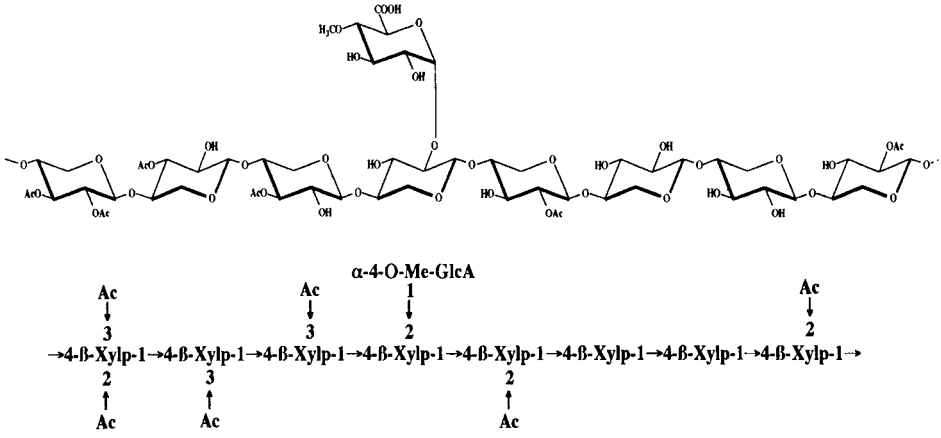


Figure 11.4-4. *O*-Acetyl-4-*O*-methyl-glucuronoxylan from hardwood.

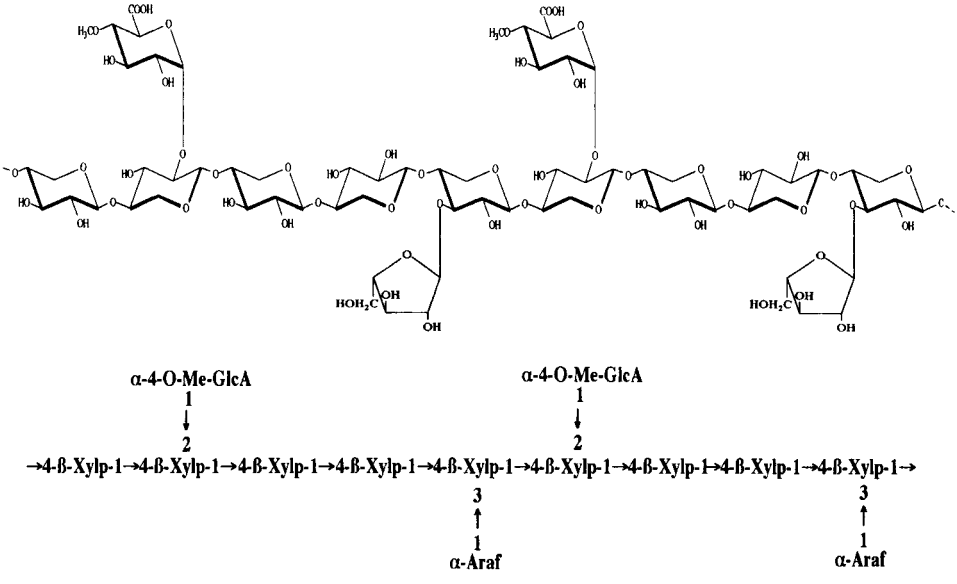


Figure 11.4-5. Arabino-4-*O-methyl-glucuronoxylan from softwood.*

11.4.4.1
The Xylanolytic Enzyme System

Because of the heterogeneity of xylan, its hydrolysis requires the action of a xylanolytic enzyme system which is composed of β-1,4-endoxylanase (E.C. 3.2.1.8), β-xylosidase (E.C. 3.2.1.37), α-L-arabinofuranosidase (E.C. 3.2.1.55), α-glucuronidase (E.C. 3.2.1.-) and acetyl xylan esterase (E.C. 3.1.1.6) activities (Table 11.4-3). The concerted action of these enzymes converts xylan to its constituent sugars (Fig. 11.4-6). Xylan-degrading enzymes have been reported to be present in marine and

Table 11.4-3. Microbial xylanolytic enzymes.

Organism	Endoxylanase	β-Xylosidase	α-L-Arabino furanosidase	α-Glucuro-nidase	Acetyl xylan esterase
Fungi					
Aspergillus awamori	+	+	+	+	+
Furiarum oxysporum	+	–	+	–	+
Tricoderma reesei	+	+	+	+	
Bacteria:					
Bacillus subtilis	+	+	+	–	+
Streptomyces olivochromogenes	+	+	+	+	+
Thermoactinomyces vulgaris	+	N.D.	+	N.D.	+
Thermoanaerobacter saccharolyticum	+	+	+	N.D.	+
Thermonospora fusca	+	+	+	N.D.	+
Thermotoga maritima	+	N.D.	N.D.	N.D.	N.D.
Thermotoga neapolitana	+	N.D.	N.D.	N.D.	N.D.
Archaea					
Thermococcus zilligii	+	N.D.	N.D.	N.D.	N.D.

N.D.: not determined

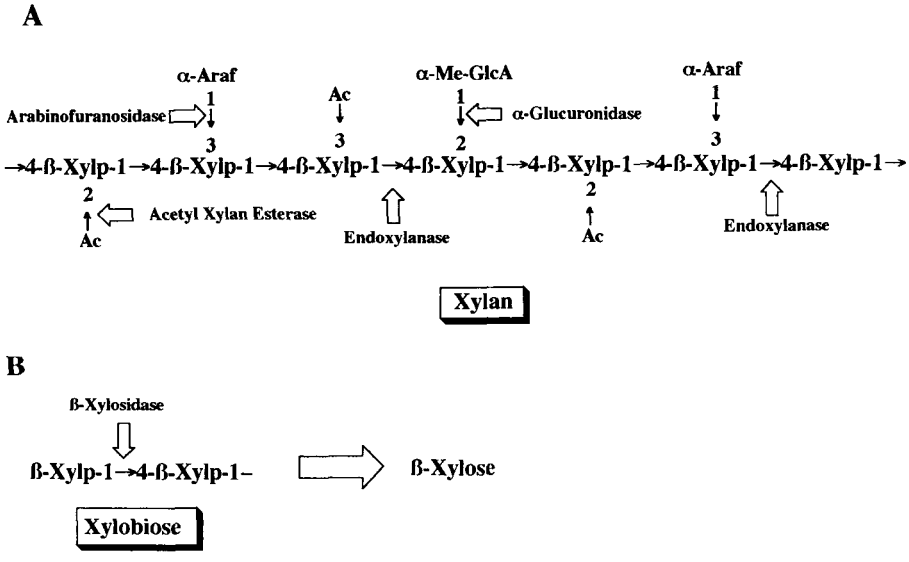


Figure 11.4-6. (A) Action of xylanolytic enzymes on an hypothetical xylan structure. (B) Action of β-xylosidase on xylobiose. Ac, acetyl residue; α-Araf, α-L-arabinofuranose; α-Me-GlcA, 4-*O*-methyl-D-glucuronic acid; β-Xylp, β-D-xylopyranose.

terrestrial bacteria, rumen and ruminant bacteria, fungi, marine algae, protozoa, snails, crustaceans, insects and seeds of terrestrial plants[144]. Among the different functions of xylanases is the utilization of xylan as a carbon and energy source, degradation of cell wall components and degradation of xylans during germination of barley[146].

11.4.4.2
Endoxylanase (1,4-β-D-Xylan Xylanohydrolase, E.C. 3.2.1.8)

β-1,4-Endoxylanase cleaves the internal glycosidic linkages of the heteroxylan backbone, resulting in a decreased DP (degree of polymerization) of the substrate (Fig. 11.4-6A). The attack of the substrate is not random, and the bonds to be hydrolyzed depend on the nature of the substrate, e.g. length, presence of substituents and degree of branching[145]. During the early course of hydrolysis of xylan the main products formed are xylooligosaccharides. As hydrolysis proceeds, these oligosaccharides are hydrolyzed to xylotriose, xylobiose and xylose[146–149]. Differentiation of endo-acting xylanases has been made according to the end products formed i.e. xylose, xylobiose and xylotriose, and/or arabinose. Thus, xylanases may be classified as non-debranching (arabinose non-liberating) or debranching (arabinose-liberating) enzymes[145, 146]. Many organisms are able to produce both debranching and non-debranching xylanases, resulting in a maximum efficiency of xylan hydrolysis[147, 150]. The production of multiple forms of xylanases has been reported for many organisms such as *Aspergillus niger* and *Fibrobacter succinogenes*[150, 151]. The endoxylanase I from *F. succinogenes* possesses debranching activity and liberates arabinose from xylan. This is followed by the action of endoxylanase II, which converts unbranched xylans to xylooligosaccharides[151]. This may indicate that the removal of the arabinose substituents, which act as a hindrance, is a requirement to permit the access of endoxylanase to the xylan backbone. This also demostrates the synergistic relation between debranching and non-debranching xylanases. Arabinose-cleaving endoxylanases have been purified from *Streptomyces roseiscleroticus*[152] and *Trichoderma koningii*[153].

11.4.4.3
β-Xylosidase (β-D-Xyloside Xylohydrolase, E.C. 3.2.1.37)

β-D-Xylosidases are exo-glycosidases that hydrolyze short xylooligosaccharides from the non-reducing end forming xylose as end product[147] (Fig. 11.4-6B). β-Xylosidases appear to be mainly cell-associated (found in the cytosol) in bacteria and yeast[134]. However, extracellular β-xylosidases have also been reported[154–156]. In the yeast *Cryptococcus albidus*, xylooligomers (xylobiose and xylotriose) enter the cells through a β-xyloside permease transport system and are converted by β-xylosidase to xylose[134]. β-Xylosidases are in most cases unable to hydrolyze xylan. However, there are some reports of xylosidases that are capable of attacking xylan and producing xylose[146]. Such exo-xylanases would have a limited hydrolysis activity towards heteroxylans, as their action would end at the branch points[140]. β-Xylosidase activity

may play a role in relieving the end product inhibition of endoxylanase. This has been reported for the enzyme system of *Thermomonospora fusca*[157]. Transferase activity is a typical feature of most β-xylosidases, resulting in products of higher molecular weight than the substrate[158]. Transfer reaction may result in the formation of both β-1,3 and β-1,4 bonds[158–160].

11.4.4.4
α-L-Arabinofuranosidase (E.C. 3.2.1.55)

α-L-Arabinofuranosidases are active against branched arabinoxylans, arabinans, arabinose-substituted xylooligosaccharides and *p*-nitrophenyl-α-L-arabinofuranoside. Their action on arabinoxylan results in the release of arabinose residues (Fig. 11.4-6A). The production of α-L-arabinofuranosidase in several actinomycetes seems to be induced among others by xylan, arabinan, and wheat bran[155, 161]. α-L-Arabinofuranosidases from *A. niger* and *S. purpurascens* are also capable of hydrolyzing both 1,3- and 1,5-α-L-arabinofuranosyl linkages in arabinan[162, 163]. The *Aspergillus niger* enzyme attacks first the α-L-1,3-linked arabinofuranosyl residues to the extent of 30% and then proceeds with a slow attack of the α-L-1,5-arabinan[162]. Synergism between α-L-arabinofuranosidase and endoxylanase has been reported. A significant increase in xylose, xylobiose and arabinose production was observed when both enzymes are used simultaneously[164].

11.4.4.5
α-Glucuronidase (E.C. 3.2.1.136)

α-D-Glucuronidases are required to hydrolyze the α-1,2 linkages between glucuronic acid and xylose residues in glucuronoxylan (Fig. 11.4-6A). Because of the lack of α-glucuronidase activity in many fungal hemicellulase preparations[135], this enzyme was not described until 1986[165]. Only a few α-glucuronidases have been purified so far; these include the enzymes from *Trichoderma reesei*, *Thermoascus aurantiacus* and *Agaricus bisporus*[166]. Thus, most of the studies on α-glucuronidases have been performed using partially purified enzymes. These enzymes release 4-*O*-methyl-glucuronic acid from 4-*O*-methyl-glucuronic acid-substituted xylooligomers, but not from the polymer[135]. Simultaneous hydrolysis of acetyl-4-*O*-methyl-glucuronoxylan with the endoxylanase from *A. oryzae* and the acetyl xylan esterase from *T. longibrachiatum* resulted in the production of non-substituted xylan fragments as well as substituted xylooligomers. These products were further treated with a β-xylosidase from *T. reesei* and an α-glucuronidase from *A. bisporus*. The α-glucuronidase was not active against these oligomers, indicating that the acetyl groups next to the glucuronosyl substituent may hinder the action of the α-glucuronidase[143].

11.4.4.6
Acetyl Xylan Esterase (E.C. 3.1.1.6)

Acetyl xylan esterase removes the *O*-acetyl substituents at the C-2 and C-3 positions of xylose residues in acetylxylan (Fig. 11.4-6A). The importance of acetyl xylan esterase in the hydrolysis of xylan was demonstrated recently[167]. It is mainly due to the fact that most of the xylan preparations used to study xylanolytic enzymes systems are alkali extracted xylans. Under these conditions mainly deacetylated xylans are obtained[168]. Nowadays, acetyl xylan esterase activity has been recognized as a part of the xylanolytic enzyme system of many organisms such as *T. reesei, T. viride, A. niger, Schizophilum commune*[169] and *Streptomyces* sp.[161]. The importance of this enzyme in the hydrolysis of xylan has been clearly demonstrated. Incubation of endoxylanases with acetylated glucuronoxylan resulted in the production of small amounts of xylose, xylobiose, xylotriose and large amounts of substituted oligomers. The addition of acetyl xylan esterase to the hydrolyzed mixture significantly increases the production of xylotriose and xylotetrose[170]. Similarly, an enzyme mixture of endoxylanase and β-xylosidase results in a limited hydrolysis of acetylated xylooligomers. The addition of acetyl xylan esterase enhanced xylose production[171]. Thus, complete hydrolysis of acetylated xylans by xylanases will require the deacetylation of the substrate by acetyl xylan esterases[167].

11.4.4.7
Mechanism of Action of Endoxylanase

Most of the studies on the mechanism of action of endoxylanase arise from the work of Biely et al.[172, 173] using the yeast *Cryptococcus albidus*. The reaction of the enzyme with 5 mM [U–^{14}C] xylotriose resulted in a constant product ratio of xylobiose to xylose throughout the reaction. However, when the concentration of [U–^{14}C] xylotriose was increased, the major product formed was xylobiose. Xylotetrose is cleaved at the middle glycosidic bond to form xylobiose. Xylopentose when present in low concentrations is converted to xylobiose and xylotriose in a ratio of 2:1. However, at higher concentrations xylotetrose is also produced. The action of endoxylanase on xylotriose, xylotetrose and xylopentose is usually accompanied by the formation of xylooligosaccharides larger than the original substrates. These studies also revealed that xylose and xylobiose can act as acceptors for the transferase reaction of xylanase. Although the acidic endoxylanase produced by *Aspergillus niger* differs from that of *C. albidus*, the mechanism of action of the enzyme is similar to the yeast enzyme. The mechanism of action of endoxylanase appears to be analogous to that reported for lysozyme and α-amylase[174].

11.4.4.8
Biotechnological Applications of Xylanases

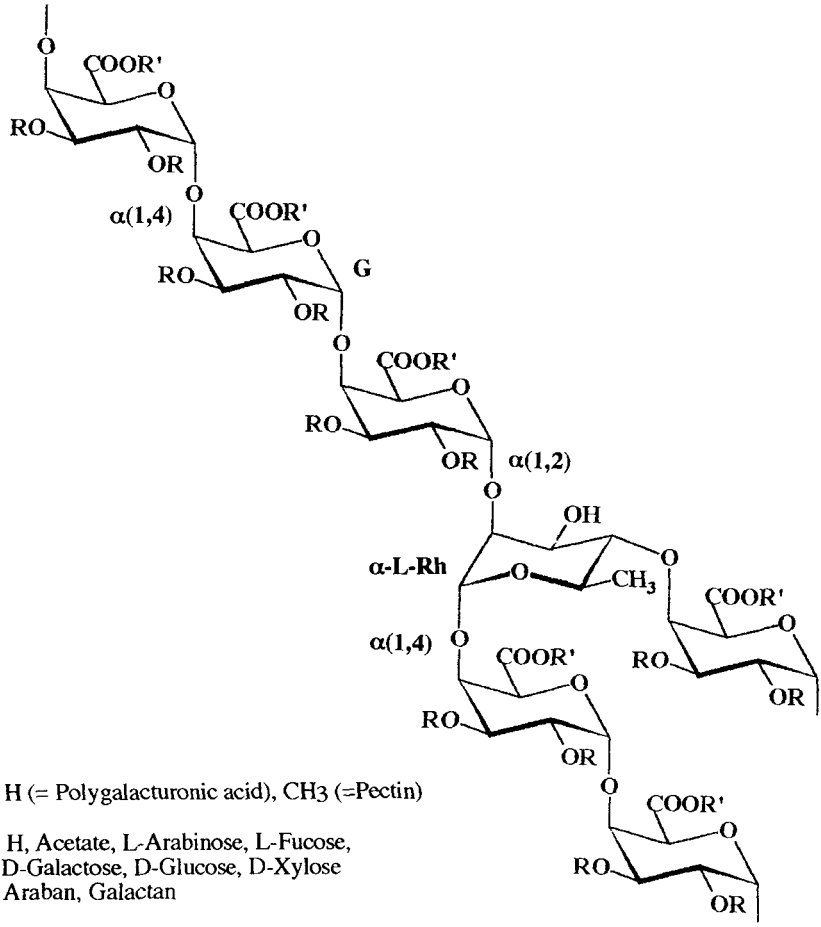
Plant polysaccharides are a major source of renewable substrates for the chemical, pharmaceutical and feed industries[129]. Xylan-degrading enzymes have considera-

ble potential in several biotechnological applications. Two main areas for the application of xylanolytic enzymes have been discussed by Biely[134]. The first is the use of xylanolytic enzymes in the presence of cellulolytic enzymes for the effective conversion of paper pulp and agricultural wastes into xylose, for the clarification of juices and must, and for the pre-treatment of cellulosic biomass to improve digestibility of ruminant feeds or to facilitate composting[130]. The second area of application involves the use of xylanolytic enzymes in the absence of cellulases[134]. Most attention has been paid to the incorporation of xylanases as pre-bleaching agents for kraft pulps. Here the use of xylanases will help in reducing the kappa numbers (measure of residual lignin) of the pulp, thus reducing the requirement for chlorine during pulp bleaching[175]. Most of the studies on the effect of xylanases in the pre-bleaching of pulp have been conducted with enzyme preparations from *Trichoderma* sp. The reduction of chlorine required during chlorination of pulp has been reported to be 35–41% for hardwoods and 10–26% for softwoods[176]. Additional applications of xylanases are as flour improvers for bakery products, in the extraction of coffee, plant oils and starch[177], for the saccharification of biomass, and in the production of fuel and chemical feedstocks[173, 178, 180].

11.4.5
Pectin

Pectic substances are widespread in the plant kingdom. The dry substance of primary cell walls of plants consists of up to 90% polysaccharides and their derivatives. These polysaccharides are composed of approximately equal parts of cellulose, hemicellulose and pectic substances. The exact proportion depends on the kind of plant (plant species) and the plant texture[181]. In fruits and vegetables, pectic substances are often found between the cells in intercellular regions. To the large, heterogenous group of pectic substances belong rhamnogalacturonans, galacturonans, arabinans, galactans and arabinogalactans[182]. Pectins are designated as rhamnogalacturonane with the structure shown in Fig. 11.4-7: molecules of galacturonic acid are linked by α-1,4 glycosidic linkages forming a helically wound chain. This chain is interrupted by rhamnose molecules which are bound by α-1,2 glycosidic linkages to the galacturonic acid[183, 184].

The number of galacturonic acid molecules varies according to the origin of the pectin. For instance, between two rhamnose molecules in citrus pectin there are 25 galacturonic acid molecules, whereas in tomato pectin there are 16 galacturonic acid molecules[181]. Pectic substances have no definite molecular weight. The molecular weight may range from 23 000 for citrus pectin to 360 000 for apple or lemon pectin[185, 186]. The break of the galacturonic acid chain by rhamnose leads to a break in the regular helical structure. In these regions, molecules are substituted to a high degree. The C-2 or C-3 atoms of the galacturonic acid and the C-4 atom of the rhamnose molecules are preferentially substituted. The substituents are acetate, L-arabinose, L-rhamnose, L-fucose, D-galactose, D-xylose or D-glucose. These substituents give to the pectin a complex and branched configuration[187, 188]. Furthermore, the main galacturonic acid chain is substituted with polymers of L-



R': H (= Polygalacturonic acid), CH3 (=Pectin)

R : H, Acetate, L-Arabinose, L-Fucose, D-Galactose, D-Glucose, D-Xylose Araban, Galactan

Rh: Rhamnose
G : D-Galacturonic acid

Figure 11.4-7. Structure of pectin.

arabinose (1,5-linked arabinan) and D-galactose (β-1,4- or β-1,3-linked galactans). Also, arabinogalactan I, which contains β-1,4 galactan, has been reported to form side chains[1]. These various side chains account for the complexity of pectic substances. The degree of substitution and the kind of substituents is dependent on the source of the pectin. In addition to the modifications on the C-2/C-3 of galacturonic acid and the C-4 of rhamnose, a large number of carboxyl groups of the galacturonic acids are esterified with methanol[189]. The degree of esterification varies with the source of the pectin. Apple pectin is esterified to the extent 80–90% and citrus pectin to 45–60%[190].

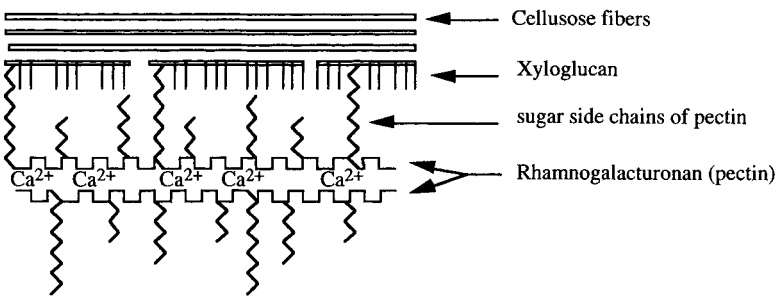


Figure 11.4-8. Structure of protopectin.

11.4.5.1
Classification of Pectic Substances

Protopectin is composed of water-insoluble pectic substances, which are fixed to the middle lamella and primary cell walls of plant cells. The neutral sugar side chain of the pectin is attached to the xyloglucan residues, which are bound to the cellulose fibers. The protopectin includes polyvalent such as calcium (Fig. 11.4-8). Protopectin is present in unripe fruits. During the maturation process of fruits or after harvesting, the protopectin is converted to soluble pectin[185]. The insolubility of protopectin may be due to the polymerization of the molecule and to the cross-linking with divalent cations[186].

Pectin (pectinate) consists of rhamnogalacturonan molecules that are modified with neutral sugar side chains. The carboxyl groups of the galacturonic acid molecules are partially esterified with methanol. The concentration of pectin in fruits varies with the degree of ripeness and the storage conditions. The average pectin concentration in fruits (not citrus fruits) varies between 0.5 and 1%[186]. The completely demethoxylated pectin is designated as polygalacturonic acid (polygalacturonate) or pectate.

11.4.5.2
Pectolytic Enzymes

Pectolytic enzymes are widespread in nature, as they have been found in plants, fungi, insects, nematodes, protozoa and bacteria. During fruit development, ripening and leaf abscission, pectin-degrading enzymes play an important role[192–195]. Furthermore, plant pectinases are important in the defensive mechanisms preventing attack of the plant by pathogenic microorganisms.

Microorganisms, especially plant pathogenic microorganisms, produce a wider spectrum of pectolytic enzymes than plants themselves. Many of these extracellular enzymes occur in multiple forms, which enhance the adaptation of the plant pathogens to different hosts[196, 197]. The most important enzyme in the plant pathogenesis process is the endo-polygalacturonase (for review see[198]). Pectinases synthesized by microorganisms also take part in symbiotic processes and in the

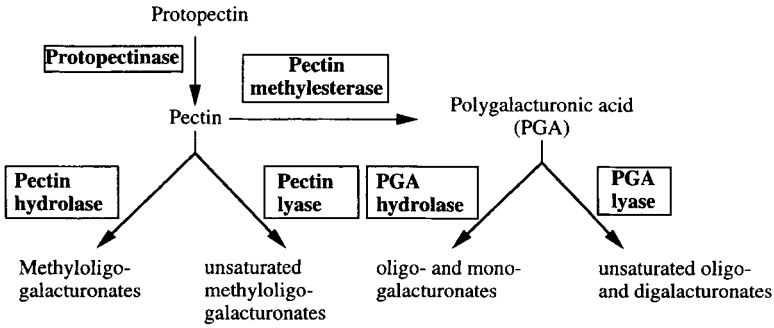


Figure 11.4-9. Action of pectolytic enzymes.

rotting of plant material. Therefore, pectolytic enzymes are widespread in pathogenic, symbiotic microoganisms, saprophytic soil bacteria and rumen bacteria. To this group belong members of the genus *Erwinia, Pseudomonas, Xanthomonas, Agrobacterium, Corynebacterium, Lactobacillus, Arthrobacter, Bacillus, Flavobacterium, Azospirillum, Actinomyces, Yersinia, Klebsiella, Clostridium, Cytophaga, Bacteroides* and *Lachnospira*[199–205, 231–234].

11.4.5.3
Classification of Pectolytic Enzymes

One can distinguish between three different types of enzymes acting on pectic substances (Fig. 11.4-9): protopectinases, which degrade protopectin, pectin methylesterases, which release methanol from the galacturonic acid, and depolymerizing enzymes. The group of depolymerizing enzymes is further divided into four subgroups according to the reaction mechanisms (hydrolases and lyases) and the substrates being used (pectin and polygalacturonic acid).

11.4.5.4
Protopectinase

Protopectinases are enzymes acting on the water-insoluble protopectin. By the action of protopectinases the protopectin is solubilized, and water-soluble highly polymerized pectin is released. These enzymes were first described by Sakai and Okushima in 1978[206]. Further investigations of protopectinases have been reported[207–209]. Protopectinases (or pectin-liberating enzymes) have two points of attack in the protopectin (Fig. 11.4-8): the polygalacturonic regions of the protopectin (A-type of protopectinases) and the sugar side chains, which connect the protopectin to the xyloglucans and to the cellulose fibers of the cell walls (B-type of protopectinases)[210].

A-type protopectinases are produced by yeast, *Kluyveromyces fragilis, Galactomyces reesei* IFO 0288 and *Trichosporon penicillatum* SNO 3. Some of these extracellular enzymes have been purified from the concentrated culture broth[211, 212]. Based on

its ability to hydrolyze the polygalacturonic acid backbone, protopectinase A is classified in the group of endo-polygalacturonases (E.C. 3.2.1.15, see also 4.2.5.1.). The protopectinase A hydrolyzes the glycosidic linkages in polygalacturonic acid if at least three unmethoxylated galacturonic acid molecules are present at a short distance. According to this, the molecular mass of pectic products increases with the increasing degree of esterification of the glucoronic acid residue[210].

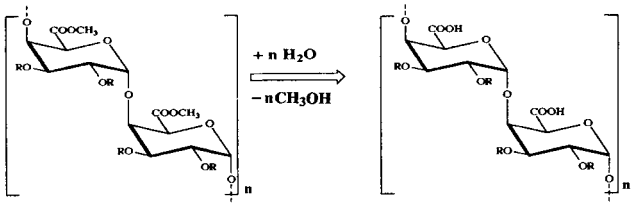
B-type protopectinases, on the other hand, are unable to degrade the polygalacturonic acid chain. These enzymes were first detected in the culture filtrate of *B. subtilis* IFO 12113 by Sakai and Ozaki in 1988[213]. Many strains of *Bacillus* species, including *B. amyloliquefaciens, B. cereus, B. circulans, B. coagulans, B. firmus, B. licheniformis, B. macerans* and *B. pumilus*, have been found to be good sources of B-type protopectinases[210]. The production of B-type enzymes is repressed in the presence of glucose and enhanced in the presence of starch and soybean flour extract containing arabinogalactan.

11.4.5.5
Pectin Methylesterase

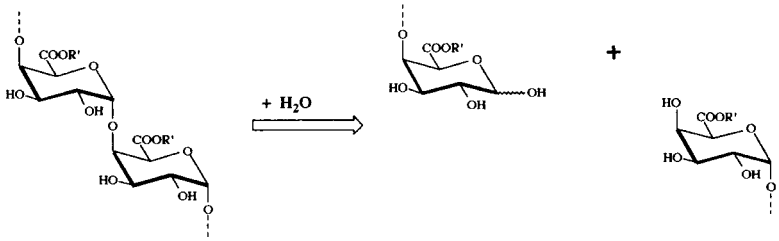
Pectin methylesterases (E.C. 3.1.1.11) deesterify the galacturonic acid methylester in pectins liberating pectic acid and methanol (Fig. 11.4-10a). The hydrolysis is characterized by high specificity and a high yield (98%)[214]. The deesterification proceeds from the reducing end of the pectin molecule in a linear mode along the chain[1]. Pectin methylesterases are produced by molds, yeasts and bacteria[185]. In general, pectin methylesterases are active in the pH range 5.0–8.0. In contrast to fungal enzymes, which are active at low pH, the bacterial esterases prefer alkaline conditions. In fruits and vegetables, especially in citrus fruits and tomatoes, high pectin methylesterase activities have also been found.

11.4.5.6
Pectin and Polygalacturonate Depolymerizing Enzymes

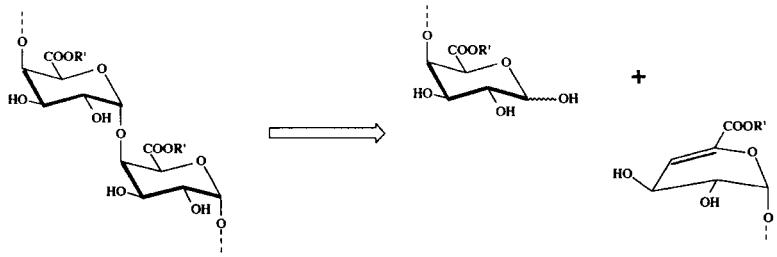
The activity of pectin-depolymerizing hydrolases, especially endoacting enzymes, can be followed by a rapid decrease in the viscosity of the pectin solutions. By the cleavage of only 2–3% of the glycosidic bonds the viscosity diminishes to about 50%. In addition, the increasing amount of reducing ends can be determined. The last stage of pectin depolymerization on an industrial scale is proved by the alcohol test. The depolymerizing reaction is complete when the addition of 50% alcohol to the reaction mixture does not lead to flocculation[214]. The activity of trans-eliminases (lyases) can be followed photometrically by measuring the UV adsorption of 4,5-dehydrogalacturonic acid at 232 nm[215].



a. Pectin methylesterase



b. Pectin and polygalacturonic acid hydrolase



c. Pectin and polygalacturonic acid lyase (R'= H: polygalacturonic acid; R'= CH_3: pectin)

Figure 11.4-10. Reaction mechanisms of pectolytic enzymes.

11.4.5.7
Pectin and Polygalacturonate Hydrolase

Pectin hydrolase and polygalacturonate hydrolase (polymethylgalacturonase, polygalacturonase) catalyze the cleavage of the polysaccharide backbone of pectin and polygalacturonate. Pectin hydrolases prefer pectin, and polygalacturonases prefer polygalacturonic acid as substrates (Fig. 11.4-10b). According to the mode of action, these enzymes can be defined as endo- or exoenzymes. Exoenzymes are able to split mono-, di- or trimers from the reducing end of the polysaccharide chain (pectin or polygalacturonic acid). Endoacting enzymes, on the other hand, attack the complex polysaccharide in the inner part of the chain backbone, resulting in a rapid decrease of viscosity of pectin- or polygalacturonate solutions. Endoacting enzymes prefer long polysaccharide chains of pectin or polygalacturonic acid. The activity decreases with decreasing chain length.

Endopolygalacturonate hydrolases (E. C. 3.2.1.15) are widespread in fungi, in most plant pathogens, in some bacteria, in plant organs and in the digestive tracts of some insects [216]. The enzyme catalyzes the random hydrolytic cleavage of α-1,4 linkages of

galacturonan and requires free carboxyl groups for their catalytic activity. The activity therefore decreases with increasing degree of esterification of the polygalacturonic acid substrate[217]. Endopolygalacturonases have been purified from several plant and microbial sources and are optimally active under acidic conditions (pH 2.5–6.5).

Most exopolygalacturonases release D-monogalacturonic acid from the non-reducing end of the chain (E.C. 3.2.1.67). The enzymes produced by *Erwinia aroideae* and *Pseudomonas* sp. (E.C. 3.2.1.82) are able to release digalacturonic acid[186, 218]. Exopolygalacturonases from fungi exhibit optimal activity between pH 4.0 and pH 6.0, whereas the enzymes from *Clostridium multifermentans* show highest activity at pH 7.2.

In addition to exo- and endopolygalacturonases a number of microorganisms produce oligogalacturonases which hydrolyze oligogalacturonate chains forming short oligomers and galacturonate. The oligogalacturonases have higher affinity to low molecular weight oligogalacturonates than to polygalacturonates. The activity decreases with increase of the chain length of the substrate. The oligogalacturonases from *Bacillus* species and *A. niger* attack the substrate from the non-reducing end, whereas the enzymes produced by *Erwinia carotovora* and *E. aroideae* hydrolyze the substrate from the reducing end[186].

11.4.5.8
Pectin and Polygalacturonate Lyase

The reaction mechanism of lyases is characterized by a trans-elimination reaction resulting in δ 4,5-unsaturated galacturonic acid molecules. The lyases are calcium dependent and attack either pectin (pectin lyases) or pectic acid (polygalacturonate lyases) from the non-reducing end (Fig. 11.4-10c).

Endopolygalacturonate lyase (E.C. 4.2.2.2) has been detected in many bacteria and some pathogenic fungi. These enzymes show highest activity under alkaline conditions in the pH range 8–10. The enzyme activity depends exclusively on the presence of calcium ions and decreases with decreasing chain length of the polygalacturonic acid.

Exopolygalacturonate lyases (E.C. 4.2.2.9) have been detected in only a few bacteria which belong to the genera of *Clostridium, Erwinia, Streptomyces* and *Fusarium*[186]. The majority of these enzymes are active under alkaline conditions (pH 8–9.5) and require calcium ions for activity. *Erwinia carotovorans* and *E. aroideae* have been found to synthesize oligogalacturonate lyase (E.C. 4.2.2.6)[219, 220]. The enzyme releases unsaturated monomers from the reducing end of the oligogalacturonate substrates.

Endopectin lyases (E.C. 4.2.2.10) are widespread in fungi and prefer long polymethylgalacturonate chains (pectin) as substrates, resulting in decreasing activity with decreasing chain length[218].

The distribution of different pectolytic enzymes in microorganisms is shown in Table 11.4-4. For review see[186, 236].

Table 11.4-4. Occurence of different pectolytic enzymes in microbes.

Organism	PME	Pectin hydrolases	Pectin lyases	PGA hydrolases	PGA lyases	Ref.
Fungi:						
Aspergillus niger	+	–	+	+	+	[664]
Aspergillus alliaceus	–	+	+	–	–	[665]
A. flavus	–	+	+	–	–	[666]
A. fumigatus	–	+	+	–	–	[666]
Botrytis cinerea	+	+	–	+	–	[667, 668]
Fusarium tricinctum	+	–	–	+	+	[669]
Yeasts:						
Candida pseudotropicalis	–	–	–	+	–	[670]
Saccharomyces vini	+	–	–	+	–	[671]
Bacteria:						
Clostridium pectinofermentans	+	–	+	+	–	[672]
C. thermosulfurogenes 4B	+	–	–	+	–	[673]
C. thermosaccharolyticum	+	–	–	+	–	[674]
Bacillus stearothermophilus	–	–	–	–	+	[675]
Corynebacterium michiganenese	–	–	+	–	+	[676]
Erwinia chrysanthemi	–	+	+	+	+	[677]
Pseudomonas marginalis	–	–	+	–	–	[678]
Streptomyces fradiae	–	–	–	–	+	[679]
Xanthomonas campestris	–	+	–	+	+	[680]

PME: Pectin methylesterase; PGA: Polygalacturonic acid.

11.4.5.9
Biotechnological Applications of Pectolytic Enzymes

Enzymes with pectolytic activity have been used since 1930 in the clarification of fruit juices. In freshly pressed apple juice, pectin acts as a stabilizing colloid for the insoluble cell debris. After hydrolysis of the pectin, the insoluble particles floc out. Also, in white wine production, a clarification process for the removal of insoluble particles suspended in the grape must is necessary[221]. The commercial enzyme preparations for industrial application may contain, as well as pectolytic enzymes, cellulases, hemicellulases, xylanases and proteases. All these enzymes solubilize the cell wall constituents to form soluble products such as galactose, mannose, rhamnose, arabinose, galacturonic acid and methanol[222, 223]. Similar processes are in use for the maceration of vegetables and the extraction of olive oil. The preincubation of sugar beet with pectolytic enzymes (1–2 h at 54 °C or 6–8 h at 18 °C) before the pressing procedure improves the yield significantly[224]. Pectin methylesterases are also used in the production of apple cider. After demethoxylation of pectin, the product formed (polygalacturonic acid) can be easily removed from the fermenting apple juice by precipitation with calcium ions[191].

Pectolytic enzymes are also involved in natural fermentation processes. The coffee seeds (coffee beans) are directly surrounded by the so-called seed coat or silver skin, followed by the endocarp (hull), the mesocarp (mucilage layer) and the exocarp (skin). One of these envelopes, the mesocarp, consists of 30% pectic substances.

Table 11.4-5. Microorganisms used for the commercial production of pectolytic enzymes.

Organism	Pectin methylesterase	Pectin hydrolase	Pectin lyase	PGA hydrolase	PGA lyase	Oligo-galacturonase
A. niger	+	+	+	+	–	+
Bacillus sp.	–	–	–	–	+	+
Penicillium sp.	+	+	+	+	–	–
Rhizopus sp.	–	–	–	+	–	–

PGA: Polygalacturonic acid.

This polysaccharide is degraded by the pectolytic enzymes that are produced by the epiphytic microbial flora of the coffee fruits, i.e. *Erwinia* and *Enterobacter* species [225]. After 1–4 days the digestion is complete and a mechanical depulping step of the coffee fruits can take place. Also, by the cocoa fermentation during the first 1–2 days, pectolytic enzymes from yeasts aid in the maceration of the cocoa pulp and the draining of the fluid. The fermentation of cocoa and coffee fruits can be enhanced by the addition of commercial enzyme preparations containing pectin-depolymerizing enzymes [235].

Protopectinases are also used in the production of pectin from mandarin orange peel. Pectin can be used as an additive in the food and cosmetic industries [226].

In all applications described above involving conventional pectolytic enzymes, the rhamnogalacturonan backbone of pectic substances is not degraded completely [227]. It has been reported that *Aspergillus aculeatus* produces an enzyme complex consisting of 10 to 15 different enzymes. This enzyme complex has the potential for the complete hydrolysis of complex polysaccharides and may support liquefaction processes with plant material, fruits or vegetables [227]. For the commercial production of pectolytic enzymes, *Aspergillus niger* or related species are mainly used. In these fermentations, low value agricultural products containing pectin are used as substrates [228–230]. Table 11.4-5 shows some of the microorganisms that are used for the industrial production of pectolytic enzymes.

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11.5
Addition of Water to C=C Bonds

Marcel Wubbolts

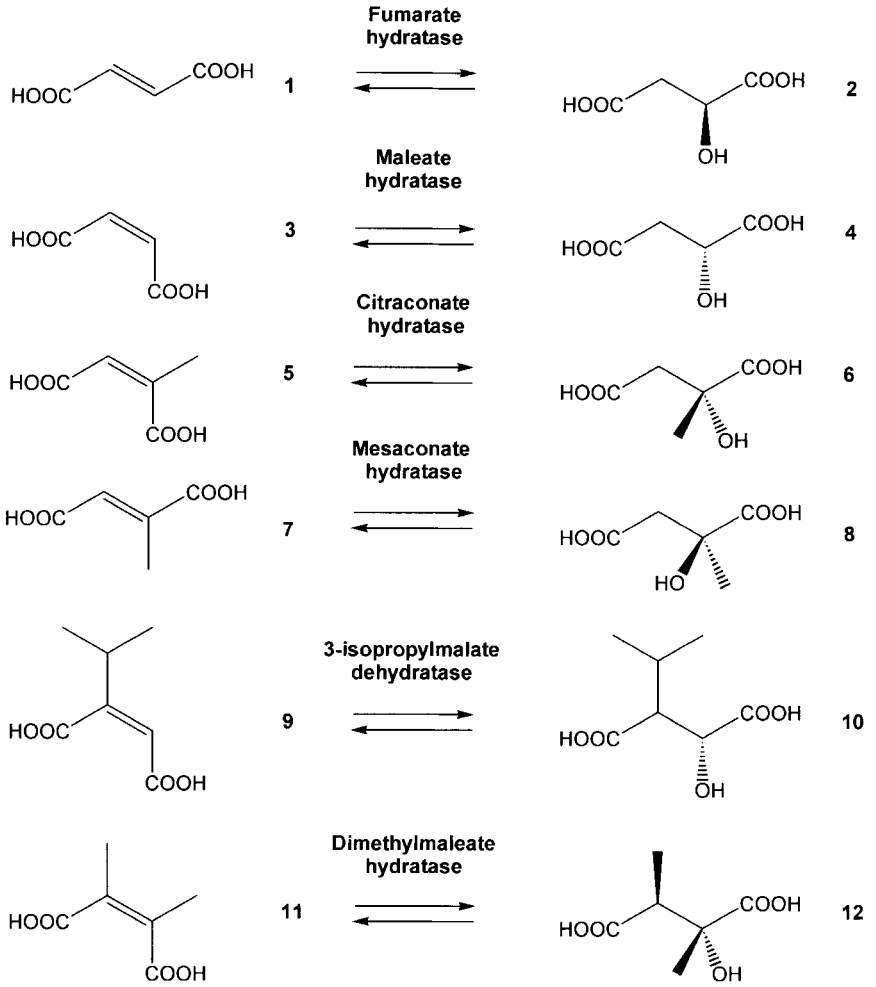
The addition of water to carbon-carbon double bonds is a reaction that is catalyzed by lyases belonging to the subclass of the hydro-lyases (E.C. 4.2.1.-), which have been grouped under the carbon-oxygen lyases. Not all members of this subgroup are capable of water addition to carbon-carbon double bonds. Nitrile hydratase (E.C. 4.2.1.84, discussed in Section 12.1) for instance, is categorized in this subclass and catalyzes the addition of water to nitriles. The nomenclature of the hydro-lyases subgroup, which contains hydratases and dehydratases, does not preclude any direction of the reaction, but rather reflects the context in which the enzyme was originally discovered.

The addition of water to carbon-carbon double bonds is very common to biology, and a large variety of enzymes from different sources representing almost a hundred different hydro-lyase types have been characterized biochemically. Hydro-lyases are for instance involved in the metabolism of a variety of carbohydrates and play a prominent role in fatty acid synthesis and degradation as well. Despite the abundant presence of hydro-lyases in nature, however, applications of these enzymes in organic chemical synthesis are not as widespread. This is mainly due to the limited availability of these enzymes and the fact that many of the enzymes cannot easily be stably maintained during catalysis.

11.5.1
Addition of Water to Alkenoic Acids

The catabolic enzyme 2-oxopent-4-enoate hydratase (E.C. 4.2.1.80) is involved in L-phenylalanine metabolism and in the degradation of a number of aromatic hydrocarbons as well [1]. It catalyzes the selective addition of water to a terminal C-C double bond of *cis*-2-hydroxypent-2,4-dienoic acid and forms 4-hydroxy-2-oxopentanoic acid. The enzyme also accepts *cis*-2-hydroxyhex-2,4-dienoic acid as a substrate, but is not active on the *trans*-isomer [2].

D-Tartaric acid dehydratase (E.C. 4.2.1.81) and the stereochemical counterpart L-tartaric acid dehydratase (E.C. 4.2.1.32) are able to catalyze the conversion of oxaloacetic acid to D- and L-tartaric acid respectively. The actual addition of water to the C-C double bond is most likely to occur at the enol tautomer, and the resulting tartaric acid has the *2S,3S* (D-stereo isomer made by E.C. 4.2.1.81) or *2R,3R* (L-tartaric acid dehydratase) configuration. Despite the stereochemistry of the reactions catalyzed, the lack of available enzyme and the instability of the enzymes in presence of oxygen [3] have hampered their application in organic synthesis thus far.



Scheme 11.5-1.

11.5.2
Addition of Water to Alkene-Dioic Acids

11.5.2.1
L- and D-Malic Acid

The production of L-malic acid (**2**) from fumaric acid (**1**) is carried out by the enzyme fumarate hydratase (E.C. 4.2.1.2), which is part of the tricarboxylic acid cycle and ubiquitous in nature (Scheme 11.5-1). The product is used in food, pharmaceutical and cosmetic industries and is produced at a multi-ten-tonne scale. Although the enzyme can be applied in isolated form, as performed by Tanabe, the use of whole cells of *Corynebacterium glutamicum* has been reported by Amino GmbH as well[4].

The conversion of fumaric to L-malic acid is brought to completion by forcing the product to precipitate as calcium salt[4].

The synthesis of D-Malic acid (**4**) from maleic acid (**3**) by maleate hydratase (E.C. 4.2.1.31) has been described as early as 1969, using an enzyme from rabbit[5]. Maleate hydratase from various other, more accessible sources such as *Pseudomonas*[6] have been used for the same purpose. The combined use of calcium-counter ions and maleate hydratase (E.C. 4.2.1.31) from *Pseudomonas pseudoalcaligenes* has been an elegant method to produce on multi-kilogram scale D-malate that in complex with calcium precipitated out from solution, thereby eliminating the reverse reaction[6]. He *et al.* used a similar enzyme from *Arthrobacter pascens* DMDC12, which is called (*R*)-2-methylmalate dehydratase, citraconate hydratase or citraconase (E.C. 4.2.1.35), to produce D-malate as well as D-citramalate or (*R*)-2-methylmalic acid (**6**) from 2-methylmaleate (**5**), using an enzyme membrane reactor system[7]. The demand for D-malate is limited: it merely serves as a general synthetic building block for chiral synthesis[6, 8] and as a resolving agent.

11.5.1.2
Substituted Malic Acids

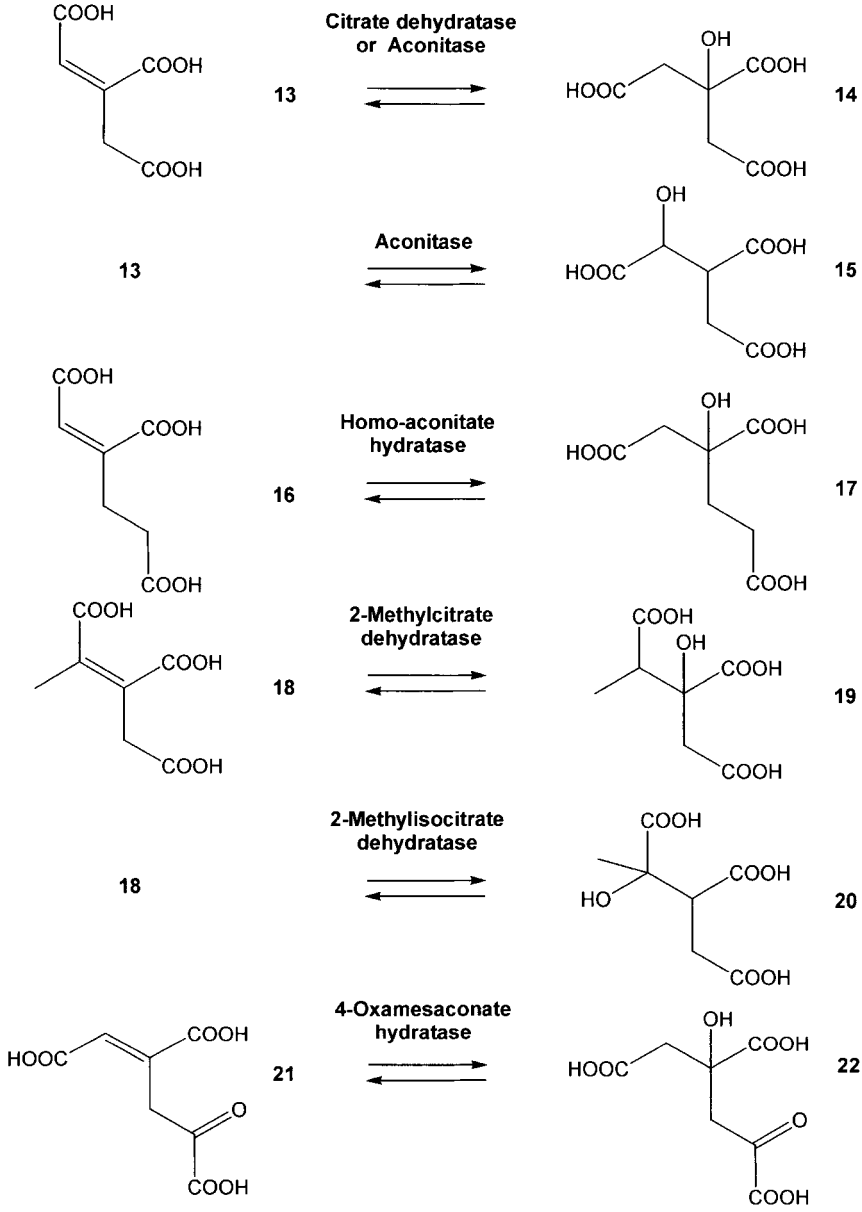
The enzyme of opposite selectivity relative to citraconase (E.C. 4.2.1.35) mentioned above, is (*S*)-2-methylmalate dehydratase or mesaconate hydratase (E.C. 4.2.1.34), which has, among others, been found in *Clostridium tetanomorphum*[9], *Pseudomonas*[10], *Citrobacter* and *Morganella*[11], and is of use to convert 2-methylfumarate (**7**) to the (*S*)-isomer of citramalate (**8**). Interestingly, both citraconase and mesaconate hydratase have a broader substrate range and are also able to produce the respective stereo-isomers of malic acid and 2-ethylmalic acid[7, 9]. The 3-*iso*-propylmalate dehydratase (E.C. 4.2.1.33) from *Neurospora crassa* and numerous other prokaryotic strains are involved in synthesis of L-valine, L-isoleucine and L-leucine. The enzyme accepts the *iso*-propyl group as a substituent during the reaction and converts 2-*iso*-propylmaleate (**9**) to 3-*iso*-propylmalate (**10**)[12].

Dimethylmaleate hydratase (E.C. 4.2.1.85) has been described as the enzyme that catalyzes the addition of water to dimethylmaleate (**11**) to yield a molecule with two chiral centers, (*2R,3S*)-2,3-dimethylmalate (**12**)[13].

11.5.3
Addition of Water to Alkene-Tricarboxylic Acids

11.5.3.1
Citric Acid and Derivatives

Other C-O lyase enzymes include aconitate hydratase or aconitase (E.C. 4.2.1.3), an enzyme that catalyzes two tricarboxylic acid cycle steps from isocitric acid to citrate (**14**)[14] or *vice versa*, via the intermediate *cis*-aconitate (**13**). Citrate dehydratase (E.C. 4.2.1.4) is only capable of converting citrate to *cis*-aconitate and does not act on isocitrate (**15**)[15].



Scheme 11.5-2.

A similar reaction is catalyzed by homoaconitate hydratase (E.C. 4.2.1.36), which is an enzyme from the L-lysine synthesis that forms homocitric acid (2-hydroxybutane-1,2,4-tricarboxylic acid, **17**) from homo-*cis*-aconitate (**16**)[16]. The enzyme 2-methylcitrate dehydratase (E.C. 4.2.1.79) catalyzes the addition of water to (*Z*)-but-2-ene-1,2,3-tricarboxylic acid (**18**) to yield 2-methylcitric acid (2-hydroxybutane-

1,2,3-tricarboxylic acid, **19**)[17]. 2-Methylisocitrate dehydratase (E.C. 4.2.1.99) from *Yarrowina lipolytica* does not accept isocitrate (**15**) as substrate, but rather acts on (*Z*)-but-2-ene-1,2,3-tricarboxylic acid (**18**) to produce 2-methylisocitrate (**20**)[18].

Lastly, 4-carboxy-2-oxohexenedioate hydratase (4-oxamesaconate hydratase, E.C. 4.2.1.83) adds water to (*E*)-4-oxobut-1-ene-1,2,4-tricarboxylic acid (**21**) and results in the formation of 2-hydroxy-4-oxobutane-1,2,4-tricarboxylic acid (**22**)[19] (Scheme 11.5-2).

11.5.4
Addition of Water to Alkynoic Acids

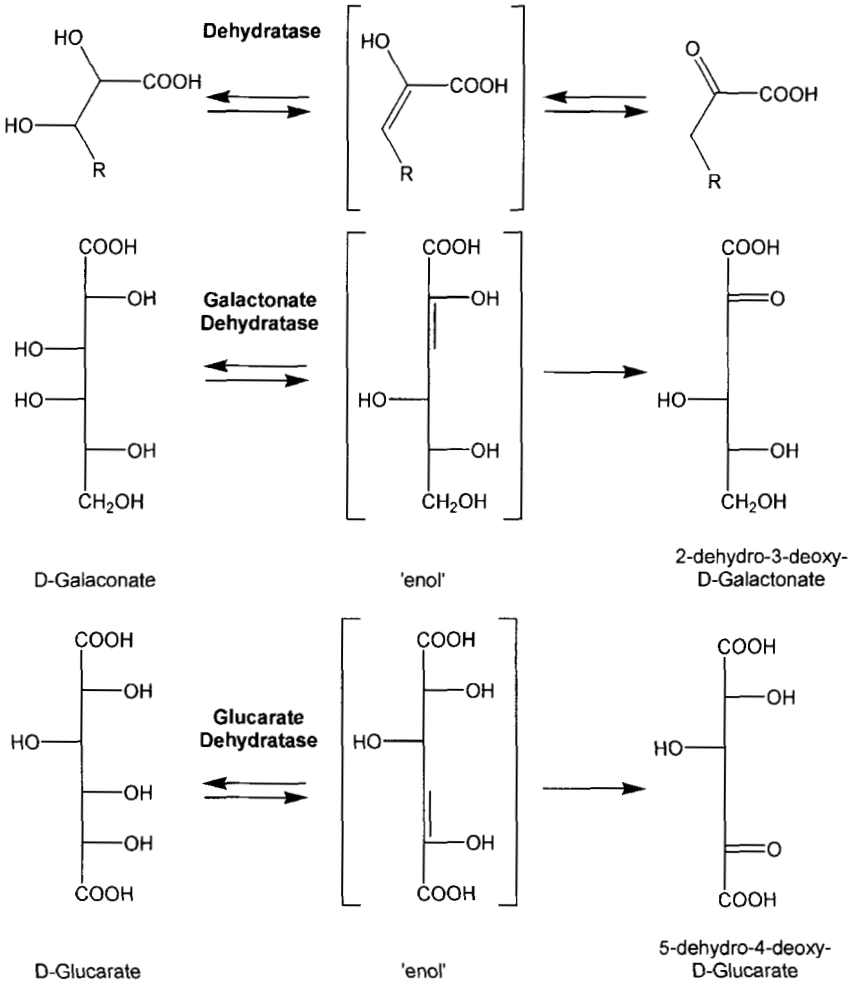
Interestingly, two enzymes have been described that catalyze the addition of water to alkynes, resulting in the formation of alkenols: acetylene carboxylate hydratase from *Pseudomonas* (E.C. 4.2.1.71), which converts propynoic acid to 3-hydroxypropenoate[20]. The latter tautomerizes to malonic semialdehyde. Acetylene dicarboxylate hydratase (E.C. 4.2.1.72) converts acetylene dicarboxylic acid to 2-hydroxyethylenedicarboxylic acid, which spontaneously decarboxylates to pyruvate[21].

11.5.5
Addition of Water to Enols

11.5.5.1
Carbohydrates: Addition of Water to 2-Keto-3-Deoxysugars

Hydro-lyases play a prominent role in the metabolism of sugars and of sugar-derived carboxylic acids in particular. The elimination/addition of water to sugar carboxylates proceeds via an enol intermediate[22], as depicted in Scheme 11.5-3. The elimination or addition of the water molecule is highly specific, and a large variety of hydrolyases have been characterized: examples include *Pseudomonas saccharophila* D-arabinoate dehydratase (E.C. 4.2.1.5)[23], *Pseudomonas* sp. and *E. coli* galactonate dehydratase (E.C. 4.2.1.6)[24], *E. coli* altronate dehydratase (E.C. 4.2.1.7)[25], *E. coli* mannonate dehydratase (E.C. 4.2.1.8)[25], L-arabinoate dehydratase (E.C. 4.2.1.25) from *Rhizobium*[26], phosphogluconate dehydratase (E.C. 4.2.1.12) from various organisms[27], gluconate dehydratase (E.C. 4.2.1.39) from various organisms[28], D-fuconate hydratase (E.C. 4.2.1.67) from *Pseudomonas* sp.[29], Mammalian L-fuconate hydratase (E.C. 4.2.1.68)[30], D-xylonate dehydratase (E.C. 4.2.1.82)[31], and fungal L-rhamnonate dehydratase (E.C. 4.2.1.90).

The elimination of water from glucarate, a 1,6-dicarboxylic hexose, by glucarate dehydratase (E.C. 4.2.1.40) results in the formation of 5-dehydro-4-deoxy-D-glucarate[32]. The reaction is however identical to that of the other dehydratases and the seemingly different specificity is only due to IUPAC rules (Scheme 11.5-3). The enzyme belongs to the enolase superfamily, and the structure of the enzyme from *Pseudomonas putida* has been resolved[33]. Similarly, galactarate dehydratase from *E. coli* (E.C. 4.2.1.42) produces 5-dehydro-4-deoxy-D-galactarate[32].

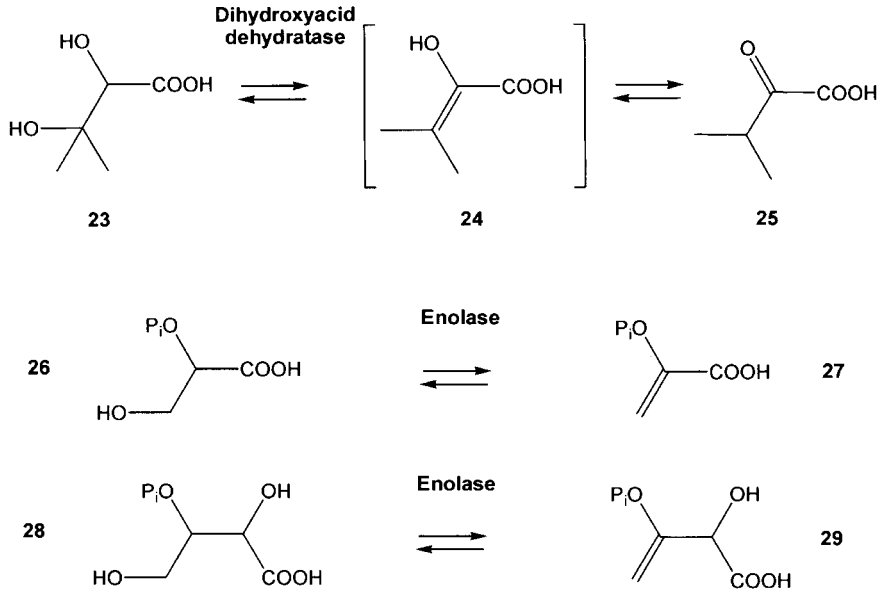


Scheme 11.5-3.

11.5.5.2

Addition/Elimination of Water with Other Enols

Dihydroxyacid dehydratase (E.C. 4.2.1.9) is a ubiquitous enzyme that is involved in the biosynthesis of the branched-chain amino acids (Ile, Leu and Val) and of pantothenic acid and coenzyme A. The enzyme catalyzes the elimination of water from 2,3-dihydroxyalkanoic acids (**23**) to 2-hydroxy-2-alkenoic acids (**24**), which tautomerize to 2-ketoalkanoic acids (**25**). The enzyme from spinach has the highest activity towards 2,3-dihydroxy-3-methylbutanoic acid (Val precursor, Scheme 11.5-4) but also accepts other substrates[34]. Thus, 2,3-dihydroxybutanoic acid, 3-cyclopropyl-2,3-dihydroxybutanoic acid as well as 2,3-dihydroxy-3-methylpentanoic acid are substrates. With the latter substrate a slight preference for (2*R*,3*S*)-2,3-dihydroxy-



Scheme 11.5-4.

3-methylpentanoate over the (2*R*,3*R*)-2,3-dihydroxy-3-methylpentanoate was observed[34].

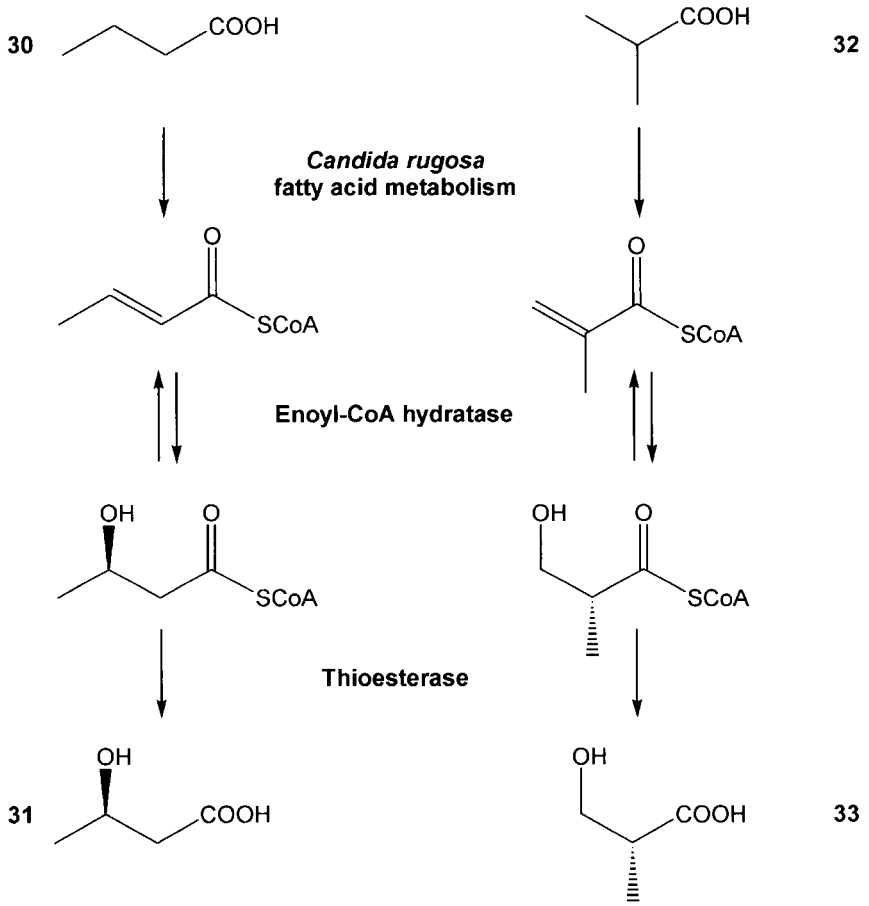
The glycolytic enzyme phosphoenolpyruvate (PEP) hydratase (enolase, E.C. 4.2.1.11) catalyzes the addition of water to 2-phospho-D-glycerate (**26**). The enzyme from *E. coli*[35] also accepts 3-phospho-D-erythronate (**28**) and thereby forms phosphoenol-4-deoxy-3-tetrulosonate (**29** in Scheme 11.5-4). Both PEP and phosphoenol-4-deoxy-3-tetrulosonate represent "fixed" enolates that can be isolated.

The enzymes 1,2-propanediol dehydratase (E.C. 4.2.1.28) and glycerol dehydratase (E.C. 4.2.1.30) from the facultative anaerobic microorganism *Klebsiella pneumoniae* [36, 37] and other sources have recently gained interest, since these enzymes can be of use for the synthesis of 1,3-propane diol (PDO) starting from glycerol. PDO is of use for the synthesis of polyesters, and Dupont is currently developing a biological production method based on fermentation. Glycerol dehydratase (E.C. 4.2.1.30) catalyzes the elimination of water from a number of polyols: ethylene glycol to acetaldehyde, glycerol to 3-hydroxypropanal and 1,2-propanediol to propionaldehyde, all of which reactions proceed via an enol intermediate.

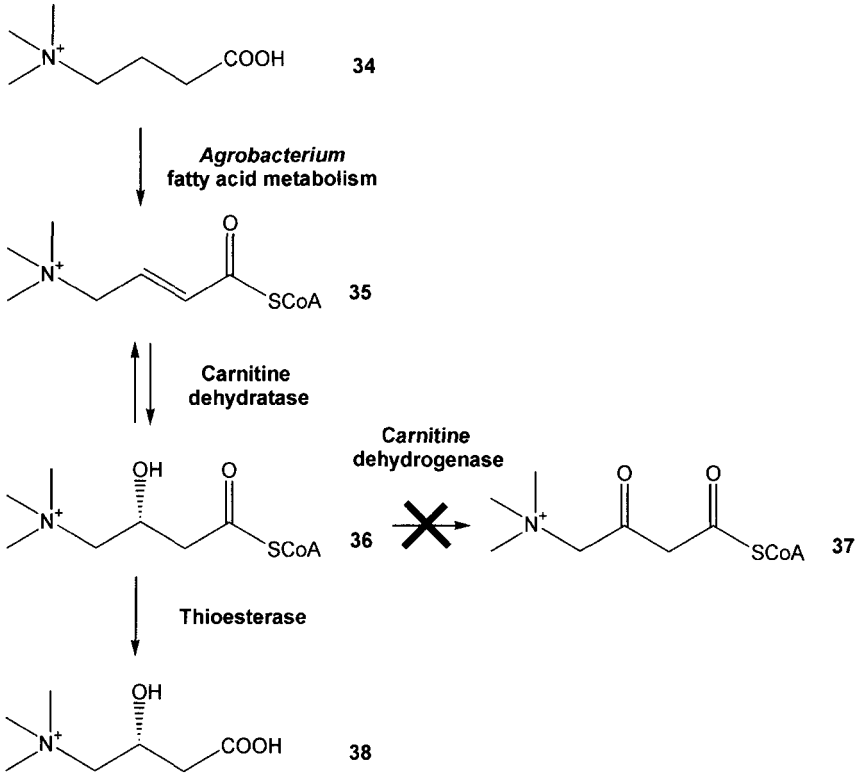
11.5.6
Addition of Water to Unsaturated Fatty Acids

11.5.6.1
CoA and ACP Coupled Fatty Acid Hydratases

Hydratases that add water to unsaturated fatty acids coupled to coenzyme A (CoA) or acyl carrier protein (ACP) cannot be used *in vitro*, and consequently have to be applied in whole-cell biotransformations. Prohibitive as this may seem to production on a commercial scale, Kanegafuchi has developed a process, making use of whole cells of *Candida rugosa*, to produce (*R*)-2-hydroxybutanoic acid (**31**) from butanoic acid (**30**) (Scheme 11.5-5). The series of reactions catalyzed by these cells include coupling of butanoic acid to CoA, desaturation of butyryl-CoA to 2-butenyl-CoA and water addition catalyzed by enolyl-CoA hydratase (enoylase, unsaturated enoyl-



Scheme 11.5-5.



Scheme 11.5-6.

coenzyme A hydratase, E.C. 4.2.1.17). Removal of the CoA group liberates the β-hydroxy acid, which is of use for the synthesis of carbapenems[38]. Similarly, the *Candida rugosa* system has been used by Kanegafuchi to produce (*R*)-2-hydroxyisobutyric acid (**33**), an intermediate for the synthesis of the ACE inhibitor Captopril from the starting compound isobutyric acid (**32**)[4, 38, 39].

The production of L-carnitine by Lonza is also carried out by whole cells that make use of CoA-coupled fatty acid degradation or the β-oxidation pathway. L-Carnitine (**38**), or (*R*)-3-hydroxy-4-trimethylaminobutyric acid, serves as a fatty acid carrier and plays an important role in the metabolism of fats. In addition to clinical applications, such as for the treatment of disorders in fat metabolism, it is also a popular over-the-counter product in fitness and anti-obesity formulations. Lonza carries out the production of L-carnitine on a multi-tonne scale, in a whole-cell process. The whole-cell process utilizes intact *Agrobacterium* cells that are fed with glucose and 4-butyrobetaine (**34**) as a precursor. The key enzyme that catalyzes the addition of water to crotonobetaine is L-carnitine dehydratase (crotonobetainyl-CoA hydratase, E.C. 4.2.1.89), which adds a water molecule to the fermentation product, crotonobetainyl-CoA (**35**, see Scheme 11.5-6). The resulting product, L-carnityl-CoA (**36**) is not oxidized to the corresponding β-keto acid **37**, since the cells lack the enzyme

carnitine dehydrogenase. Instead, the CoA coenzyme is hydrolyzed off by a thioesterase resulting in the release of L-carnitine (**38**) in the medium[40].

Despite the fact that numerous enzymes have been characterized that catalyze the addition of water to unsaturated fatty acids that are coupled to CoA or ACP, such as methylglucatonyl-CoA hydratase (E.C. 4.2.1.18), lactoyl-CoA dehydratase (E.C. 4.2.1.54), 3-hydroxybutyryl-CoA dehydratase (E.C. 4.2.1.55), itaconyl-CoA dehydratase (E.C. 4.2.1.56), isohexenylglutaconyl-CoA hydratase (E.C. 4.2.1.57), farnesyl-CoA dehydratase (E.C. 4.2.1.57), long-chain enoyl-CoA hydratase (E.C. 4.2.1.74), 3-hydroxydecanoyl-ACP dehydratase (E.C. 4.2.1.60) and 3-hydroxypalmitoyl-ACP dehydratase (E.C. 4.2.1.61), these enzymes are seldomly applied in organic synthesis.

An unusual coenzyme A-coupled hydratase reaction occurs during the anaerobic degradation of benzoic acid by *Thauera aromatica*, where cyclohexa-1,5,–diene-1-carboxylate CoA hydratase (E.C. 4.2.1.100) adds a water molecule to the cyclohexadiene functionality, resulting in the formation of 6-hydroxycyclohex-1-enecarbonyl CoA[41].

11.5.6.2
Hydratases Acting on Free Fatty Acids

The enzyme oleate hydratase (E.C. 4.2.1.53) from *Pseudomonas* catalyzes the elimination of water from (*R*)-10-hydroxystearate or the addition of water to a number of free unsaturated fatty acids, yielding (*R*)-10-hydroxy fatty acids. Substrates that have been identified include linoleic acid, oleic acid and palmitoleic acid, which are converted to the corresponding 10-hydroxy-fatty acids[42].

11.5.7
Addition of Water to Steroids

Hydratation of unsaturated carbon-carbon bonds in the steroid nucleus has been ascribed to hydrolyases such as 5-α-hydroxysteroid dehydratase (E.C. 4.2.1.62) from *Saccharomyces cerevisiae*, which catalyzes the interconversion of 5-α-ergosta-7,22-diene-3-β-5-diol and ergosterol[43]. Similarly, the formation of 16-α-hydroprogesterone from 16-dehydroprogesterone is catalyzed by 16-dehydroprogesterone dehydratase. The same enzyme catalyzes the addition of water to 16-α-hydroxyprogesterone or 16-α-hydroxy-pregnenolone, yielding the corresponding 16,17-didehydroprogesterone and 16,17-didehydropregnenolone (E.C. 4.2.1.86 or E.C. 4.2.1.98[44]).

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12
Hydrolysis and Formation of C-N Bonds

12.1
Hydrolysis of Nitriles

Birgit Schulze

12.1.1
Introduction

Organic nitriles are used extensively industrially as precursors for the production of a wide variety of amides and acids by chemical synthesis. In recent years, considerable attention has been paid to enzymatic hydrolysis of nitriles as an alternative route to the chemical synthesis of amides and carboxylic acids. Conventional chemical conversion of nitriles suffers from several disadvantages, including the requirement for highly acidic or basic reaction conditions, high energy consumption, formation of undesirable by-products, low yields and environmental problems due to the generation of waste salts.

Biocatalysis, on the other hand, may be performed under mild conditions (low temperatures, neutral pH) thus affording high conversion yields and selective hydrolysis of the -CN functionality of compounds containing acid or base labile groups. Furthermore, enzyme hydrolysis is, in some instances, enantio- and/or regioselective.

This chapter does not aim to give a complete treatise on the extensive literature on nitrile bioconversions but rather aims at presenting a brief overview of enzymatic nitrile hydrolysis with a selection of examples. Several reviews on the bioconversion of organic nitriles and its potential technological application have been published[1–7].

12.1.2
Types of Nitrile Hydrolyzing Enzymes

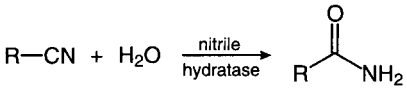
12.1.2.1
Enzymatic Hydrolysis of Organic Nitriles

A variety of organic nitriles, such as cyanoglycosides and cyanolipids, occur naturally in biological material[1, 3]. It has been shown that nitrile-hydrolyzing activity is widespread in bacteria and fungi but has recently also been identified in insects and humans[8]. In microorganisms the hydrolysis of organo-nitriles (hereafter denoted as nitriles) is effected by two distinct enzymes, nitrilases or nitrile hydratases.

Nitrilase (E.C. 3.5.5.1.) catalyzes the hydrolysis of nitriles to the corresponding acids and ammonia in a one step reaction without the formation of a free amide intermediate:

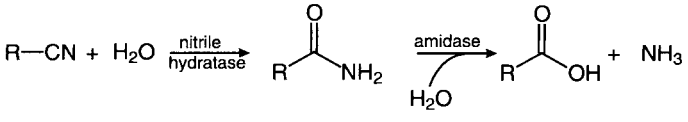
Scheme 12.1-1.

whereas nitrile hydratase (E.C. 4.2.1.84) catalyzes the hydrolysis of nitriles to the corresponding amides:



Scheme 12.1-2.

Microorganisms which produce a nitrile hydratase also seem to synthesize one or more amidase enzymes [linear amide hydrolase (E.C. 3.5.1)] thus enabling them to hydrolyze nitriles to the corresponding acids in a two-step reaction:



Scheme 12.1-3.

Nitrilases and nitrile hydratases are distinct enzymes, apparently differing both with respect to prosthetic groups and reaction mechanisms.

12.1.2.1.1 **Nitrile Hydratases**

In recent years nitrile hydratases have been studied intensively. Enzymes from a wide range of microorganisms have been isolated and characterized and the corresponding genes have been cloned and overexpressed. Nitrile hydratases can be subdivided into two classes: enzymes requiring Fe^{3+} (for example: from *Rhodococcus* sp.[9–12], *Pseudomonas chlororaphis* B23[13] and *Comamonas testosteroni* NI1[14]) and

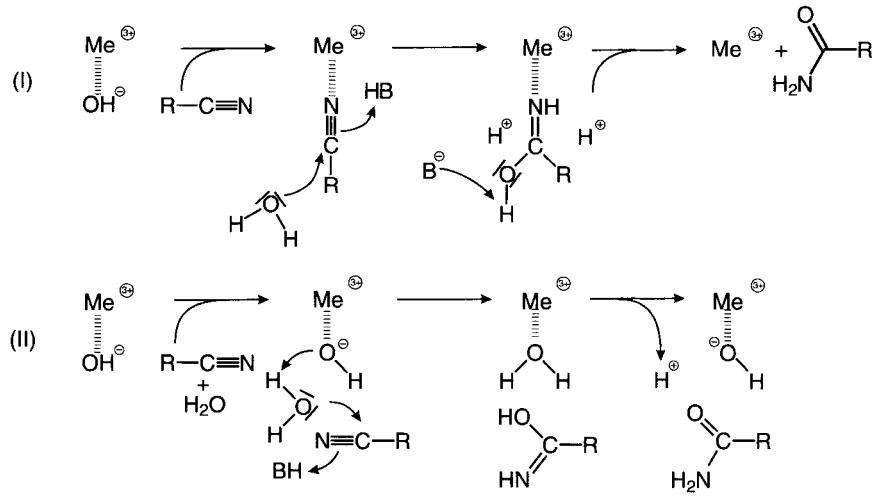


Figure 12.1-1. Two of the mechanisms discussed for nitrile hydratase catalysis[24–27]. (I) Direct coordination of the substrate to the metal ion in the active site. (II) Attack of a water molecule activated by a metal-bound hydroxide ion.

enzymes requiring Co^{3+} (for example: *Rhodococcus rhodocrous* J1[15,16] and *Pseudomonas putida* NRRL 18668[17]) for catalysis. The metal clusters of the former group have been studied intensely by EPR, ENDOR, EXAFS and other spectrophotometric techniques, revealing a unique coordination of a non-heme iron cluster[18–24].

Nitrile hydratases show a high degree of homology. They consist of from two up to twenty subunits[2] containing one metal ion per α,β-dimer with the only exception described to date being the nitrile hydratase from *P. chlororaphis* which appears to be a homotetramer[13].

The first X-ray structure of a nitrile hydratase was determined for the enzyme from *Rhodococcus* sp. R312[25]. In this study an $\alpha_2\beta_2$-tetramer conformation has been found in the native enzyme. Characterization of the highly related *Rhodococcus* sp. N-771 nitrile hydratase, however, revealed a dimeric species in solution for this enzyme[12].

Based on the X-ray structure mechanisms have been proposed which are depicted in Fig. 12.1-1[25–27]. Earlier studies suggested the involvement of the cofactor pyrroloquinoline quinone (PQQ)[13]. X-ray studies and spectral characterization[24, 25] have, however, recently discarded this.

12.1.2.1.2 Nitrilases

Nitrilases have been studied less than the nitrile hydratases. The enzymes appear as homomultimers, exhibiting a wide range of molecular masses. The reaction mechanism depicted in Fig. 12.1-2 has been proposed recently by Kobayashi et al.[28]. Several nitrilases have been found to be inhibited by reagents which bind to thiol groups, indicating that sulfhydryl groups are essential for the catalytic activity of

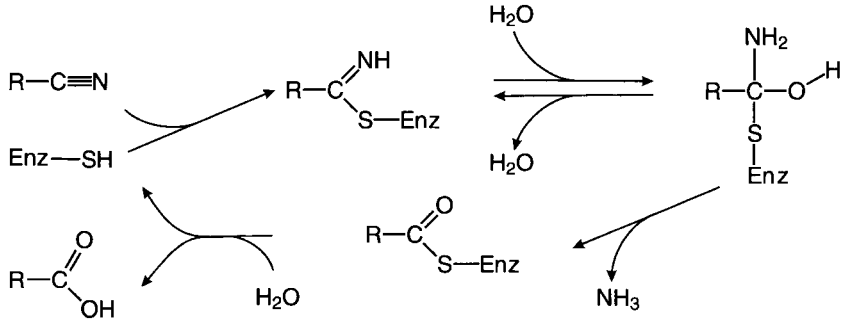


Figure 12.1-2. Reaction mechanism proposed for nitrilase catalysis [28].

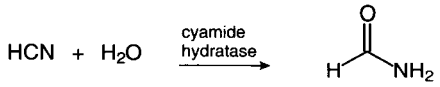
these enzymes. This has been confirmed by the work of Kobayashi et al. who cloned the nitrilase gene from *Rhodococcus rhodocrous* J1 and have proved that Cys 165 is crucial for nitrilase activity. The group also found that the nitrilase does catalyze the formation and hydrolysis of amides, although with lower activity [28].

All the nitrile hydrolyzing enzymes described so far are intracellular and differ considerably with respect to substrate specificity, stereoselectivity, molecular mass and substrate and product inhibition characteristics.

12.1.2.2
Enzymatic Hydrolysis of Cyanide

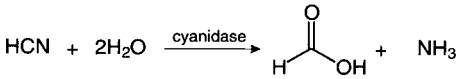
Microorganisms appear to have evolved separate metabolic pathways for the hydrolysis of inorganic cyanide. Thus most nitrilases and nitrile hydratases investigated so far do not display activity towards cyanide. However recently, as a first exception to this, a nitrilase has been reported that hydrolyses potassium cyanide as well as organic nitriles [29, 30].

Enzymes catalyzing the hydrolysis of cyanide had already been identified earlier. Here too, two different types can be distinguished. The enzyme cyanide hydratase [formamide hydrolase (E.C. 4.2.1.66)] can be found in various fungi [31]. It catalyzes the hydration of cyanide to formamide:



Scheme 12.1-4.

A direct hydrolytic activity on cyanide, yielding formate and ammonia without forming formamide as an intermediate, has been identified in different bacteria [32,33].



Scheme 12.1-5.

This new type of enzyme, tentatively named 'cyanidase', has also recently been cloned, overexpressed and characterized[34, 35]. Cyanidase tolerates high cyanide concentrations and is able to deplete cyanide in aqueous solutions to less than 0.01 ppm.

Cyanide hydrolyzing enzymes (i.e. reaction schemes 12.1-4 and 12.1-5) have, with the exception mentioned above, not been reported to hydrolyze organic nitriles and thus appear to be highly specific for inorganic cyanide. Therefore, they are mainly of interest in waste water treatment as a biological alternative to conventional chemical detoxification of cyanide by alkaline chlorination and will not be treated further.

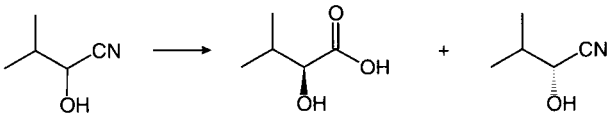
Comprehensive reviews on biological cyanide transformations may be found in the literature[36, 37]. Reviews on microbial cyanide metabolism have been published by Knowles[38, 39] and Knowles and Bunch[40].

12.1.3
Examples of Enzymatic Nitrile Hydrolysis

12.1.3.1
Enantioselective Hydrolysis of Nitriles

Several groups of investigators have reported on the production of optically active acids from racemic mixtures of nitriles. A comprehensive overview has recently been presented[41].

Fukuda and coworkers have described one of the first applications of the enantioselective hydrolysis of nitriles (Scheme 12.1-6). Using a whole cell biocatalyst optically pure α-hydroxy acids (L-α-hydroxyisovaleric acid and L-α-hydroxyisocaproic acid) have been prepared from the racemates of the corresponding α-hydroxynitriles[42].



Scheme 12.1-6.

In recent years, the enantioselective hydrolysis of nitriles has been studied in more detail. Whereas in the past only whole cell catalysts had been investigated, it is now possible to assign the activities to specific enzymes occurring in the cell. These enzymes are nitrilases, nitrile hydratases and/or amidases.

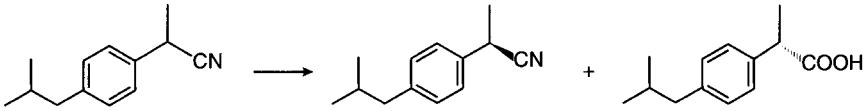
12.1.3.1.1 **Enantioselective Nitrilases**

First indications of stereoselective nitrilases have been given by Macadam and Knowles describing the production of L-alanine from racemic α-aminopropionitrile by a stereoselective nitrilase produced by an *Acinetobacter* sp.[43].

Bhalla et al.[44] reported the stereoselective hydrolysis of α-aminonitriles by a

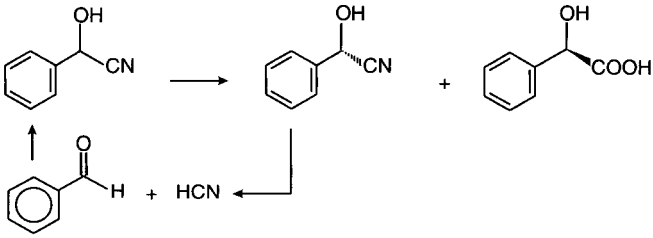
nitrilase from *Rhodococcus rhodochrous* PA-34. The nitrilase exhibited relaxed substrate specificity hydrolyzing both mono- and dinitriles and its stereospecificity differs from that of the *Acinetobacter* sp. since it produced D-alanine from D,L-α-aminopropionitrile.

Enzymatic production of *(S)*-(+)-ibuprofen, an anti-inflammatory drug, from racemic 2-(4'-isobutylphenyl)propionitrile using various bacterial strains has been published by Yamamoto et al. (Scheme 12.1-7)[45, 46]. One of the strains, *Acinetobacter* sp. AK226 produced *(S)*-(+)-ibuprofen by means of a nitrilase in whole cell experiments. High optical purity of the product (about 95 % ee) was obtained at a low percentage of hydrolysis but the optical purity of the product decreased at the later stages of the reaction. This was in accordance with other results which showed that *(R)*-(-)-Ibu-CN was indeed also hydrolyzed by the nitrilase, although at a 108-fold lower rate than the *(S)*-nitrile. Also *Rhodococcus butanica* ATCC 21 197 has been shown to produce a nitrilase suitable for the enantioselective hydrolysis of racemic 2-aryl-propionitriles; however, selectivities were rather low[47].



Scheme 12.1-7.

An enantioselective nitrilase has also been shown to be applicable in the dynamic kinetic resolution of mandelonitrile. Using the nitrilase produced *by Alcaligenes faecalis* ATCC 8750 Yamamoto et al. showed that they could derive *(R)*-(-)-mandelic acid from mandelonitrile in 91% yield with an ee of 100%. Under the reaction conditions used non-reacting *(S)*-mandelonitrile undergoes spontaneous racemization leading to the high yield (see Scheme 12.1-8)[48]. Currently *(R)*-mandelic acid and *(R)*-chloromandelic acid are produced using nitrilases on an industrial scale by the Mitsubishi Rayon Corp.

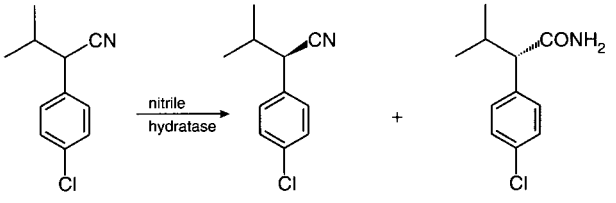


Scheme 12.1-8.

12.1.3.1.2 Nitrile Hydratases

Enantioselective hydrolysis of nitriles by the nitrile hydratase/amidase system has often been attributed to the combination of a non-selective nitrile hydratase and a selective amidase. However, more recently several enantioselective nitrile hydratases have also been identified and studied in detail. For example, a nitrile hydratase from

Pseudomonas putida NRRL-18668 has been found to hydrate 2-(4-chlorophenyl)-3-methylbutyronitrile with high enantioselectivity (Scheme 12.1-9). The whole cell preparation was found to transform the *(S)*-enantiomer preferentially until approximately 90% has been consumed. Successively the *(R)*-enantiomer also reacted but at a 6-fold lower rate[49]. Using a purified enzyme preparation on the enantiomerically pure substrates the reaction rate towards the *(S)*-enantiomer has been determined to be more than 50 times higher than for the *(R)*-enantiomer[17]. Recently the enzyme has also been cloned and overexpessed in *E.coli*[50] and *Pichia pastoris*[51]. The whole cells from *Pseudomonas putida* NRRL-18668 have also been investigated in the hydrolysis of α-substituted arylpropionitiriles. A stereoselective hydrolysis was found. However, in these cases the enantioselectivity must be attributed to the combined reactions of the hydratase and the amidase[49]. This is also the case for several other whole cell catalysts containing the hydratase/amidase system. Hence the enantioselective hydrolysis of α-substituted phenylacetonitriles by *Rhodococcus* sp. AJ 270[52] and by *Rhodococcus* sp. (SP361)[53] is based on the highly *(S)*-selective amidases. However, evidence for enantioselective hydratases has also been provided by studying the transformations using a whole cell catalyst in the presence of amidase inhibitors. Thus, the hydratase of *Rhodococcus equi* exhibits a preference for *(S)*-nitriles whilst the hydratase from *Agrobacterium tumefaciens* is selective towards the *(R)*-nitriles[54, 55].



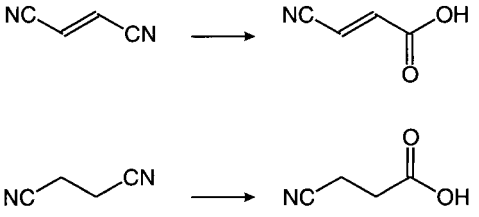
Scheme 12.1-9.

12.1.3.2
Monohydrolysis of Dinitriles

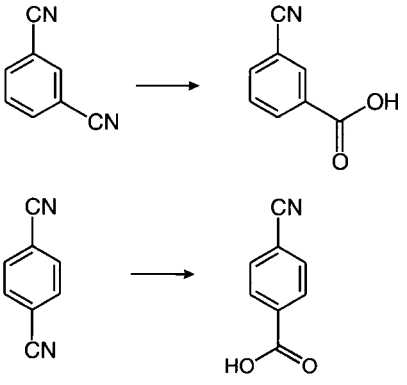
Several investigators using enzymes of different microbial origin have reported on the monohydrolysis of dinitriles to the corresponding cyano-carboxylic acids in high yields. Selective hydrolysis of one cyano group of a dinitrile is very difficult to carry out by chemical hydrolysis and is, therefore, an intriguing aspect of nitrile bioconversion.

Using whole cells of *Rhodococcus rodochrous* NCIB 11216 Bergis-Garber and Gutman demonstrated complete monohydrolysis of fumaronitrile and almost complete monohydrolysis of succinonitrile, 1,3-dicyanobenzene and 1,4-dicyanobenzene (Scheme 12.1-10, 12.1-11)[56, 57].

Analysis of the reaction products showed that during conversion of succinonitrile into 3-cyanopropionic acid, succinamic acid ($H_2NOC\text{-}CH_2\text{-}CH_2\text{-}COOH$) was detected as a free intermediate in the reaction mixture, suggesting that enzymatic activities other than nitrilases (i.e. nitrile hydratase) were present in the cell

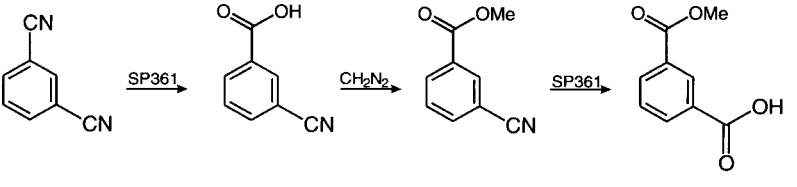


Scheme 12.1-10.



Scheme 12.1-11.

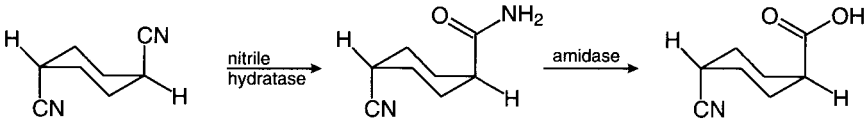
preparation. In parallel experiments with the same bacterial strain glutaro-, adipo- and pimelodinitriles were hydrolyzed further to glutaric, adipic and pimelic acid, respectively[57]. Kobayashi et al.[58] on the other hand obtained complete conversion of glutarodinitrile into cyanobutyric acid without any formation of glutaric acid using a nitrilase from another strain of *Rhodococcus* (*Rodococcus rodochrous* K22). Turner et al.[59] have studied the hydrolysis of aromatic dinitriles using an immobilized preparation of *Rhodococcus* sp. (Novo SP 361). Only after esterification of the carboxylic acid formed was the second nitrile group hydrolyzed by repetitive use of the catalyst (Scheme 12.1-12).



Scheme 12.1-12.

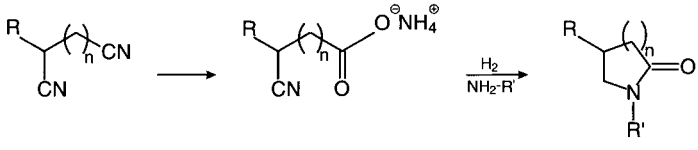
The chemoselecivity of nitrile hydrolyzing enzymes has also been used by Tani and coworkers to examine the formation of *trans*-4-cyanocyclohexane-1-carboxylic acid (*t*-MCC) from *trans*-1,4-dicyanocyclohexane (*t*-DCC) using a resting cell system of *Corynebacterium* sp. C5 (Scheme 12.1-13)[60]. The reaction was shown to be

catalyzed by the sequential action of a nitrile hydratase and an amidase, both of which were purified and characterized[61]. The nitrile hydratase exclusively hydrolyzed *trans*-1,4-dicyanocyclohexane (*t*-DCC) into *trans*-4-cyanocyclohexane-1-carboxyamide (*t*-MCMA), which could be detected in the reaction mixture[61]. The nitrile hydratase did not attack the nitrile groups in *t*-MCC and *t*-MCMA. This conversion has also been described using the nitrile hydratase from *Rhodococcus rhodochrous* AJ270 giving a 99% yield[62,63].



Scheme 12.1-13.

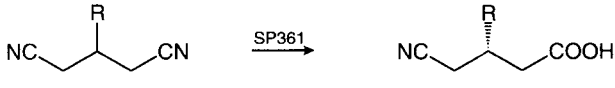
In addition to the outstanding chemoselectivity, high regioselectivity can also be found in the hydrolysis of dinitriles and has been used for example in the chemoenzymatic production of lactams from aliphatic α,ω-dinitriles. Using a nitrilase from *Acidovorax facilis* or a nitrile hydratase/amidase system from *Comamonas testosteroni* high yields of the lactams have been achieved (see Scheme 12.1-14)[64].



Scheme 12.1-14.

The chemo- and enantioselectivity of nitrile-hydrolyzing enzymes gives rise to products of high optical purity with a theoretical yield of 100%, when starting from prochiral substrates. Thus several whole cell catalysts have been studied in the conversion of 3-substituted glutaronitriles. The enantioselectivity was found to be highly dependent on the substituent at the 3-position (Scheme 12.1-15, Scheme 12.1-16)[53, 65]. Using this technology *(R)*-4-hydroxy-5-cyanopentene, a precursor for the protected lactone moiety of the δ-lactone pharmacophore of the mevinic acids, becomes available in high yields[66].

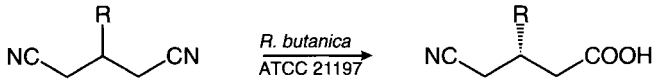
Recently, an industrial application of the chemoselective hydration of a dinitrile, adiponitrile, has been introduced. A *Pseudomonas chlororaphis* B23 nitrile hydratase



R= OBn	e.e. = 83%	73% yield
OBz	e.e. = 84%	25% yield
OH	e.e. = 22%	52% yield
OMEM*	e.e. = 61%	19% yield
OAc	e.e. = 0	45% yield

*MEM = methoxyethoxymethyl

Scheme 12.1-15.



R= Bn	e.e. = 29%	59% yield
OBn	e.e. = 90%	68% yield
OBz	e.e. = > 99%	71% yield

Scheme 12.1-16.

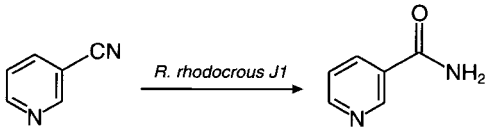
immobilized in alginate beads is used for the production of 5-cyanovaleramide. The biocatalyst is extremely stable and has been used in almost 60 consecutive batches producing more than 13 metric tons in the production of the precursor of a new herbicide[67].

12.1.3.3
Substrate and Product Inhibition of Nitrile Hydrolysis

Substrate and/or product inhibition may seriously reduce the productivity of nitrile-hydrolyzing enzymes. Already nitrile concentrations higher than 200–500 mM have been reported to be inhibitory, often causing rapid and irreversible inactivation of the biocatalyst[68–74]. Substrate inhibition may be overcome by running the enzymatic reaction constantly at a low substrate concentration using periodic or continuous feeding of the substrate. Product inhibition/inactivation, on the other hand, is considerably more difficult to tackle in a large scale industrial process and may prevent implementation of enzymatic hydrolysis for a particular reaction. Thus it appears that the success of the commercial acrylamide process of the Mitsubishi Rayon Corp. (the former Nitto Corp.) is the result of extensive and elegant efforts within the areas of process optimization and the development of improved biocatalysts which are less susceptible to product inhibition. Currently the acrylamide production is run optimally using a highly efficient nitrile hydratase catalyst at low temperature (5–10 °C) thereby avoiding substrate inhibition which occurs at higher temperatures[2, 75]. For details see Sect. 12.1.3.5.

The same whole cell catalyst can be used in the hydration of 3-cyanopyridine to nicotinamide (Scheme 12.1-17). This vitamin, broadly applied in animal feeding, is currently produced biocatalytically on an industrial scale (> 3000 t/a) by the Lonza AG. For this substrate Yamada and Kobayashi showed that the whole cell catalyst of *Rhodococcus rhodocrous* J1, containing a nitrile hydratase induced with crotonamide, can even tolerate substrate concentrations up to 12 M[2] (see Fig. 12.1-3).

Mauger et al. also succeeded in achieving high final product concentrations of various amides when using the *Rhodococcus rhodochrous* J1 catalyst (see Table 12.1-1).



Scheme 12.1-17.

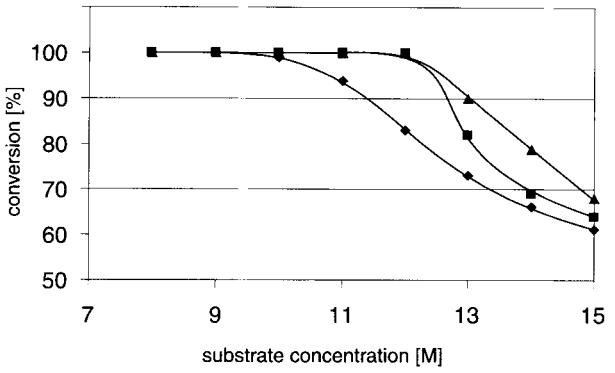


Figure 12.1-3. Conversion of 3-cyanopyridine after 5 (◆), 9 (■) and 22 h (▲) of incubation at various substrate concentrations.

Table 12.1-1. Nitriles hydrolyzed by *Rhodococcus rhodocrous* J1 [2, 76].

Substrate	Amide	Product concentration ($g\ L^{-1}$)
3-Cyanopyridine	nicotinamide	1465
4-Cyanopyridine	isonicotinamde	1099
2,6-Difluorobenzonitrile	2,6-difluorobezmide	306
2-Cyanopyrazine	pyrazinamide	985
2-Cyanopyridine	picolinamide	977
2-Cyanothiophen	2-thiophencarboxamide	210
3-Indolylacetonitrile	indole-3-acetamide	1045
Benzonitrile	benzamide	489
2-Cyanofuran	furanecarboxamide	522

The hydrations were carried out either at low substrate concentrations with slow feeding of the substrate (for example: benzonitrile, 2,6-difluorobenzonitrile and 3-indoleacetonitrile) or, in the case of less toxic substrates, by direct incubation at high substrate concentrations (for example: 3-indolylacetonitrile and 2-cyanopyrazine [2, 76]).

In addition, high substrate levels have been used in the industrial production of 5-cyanovaleramide (see Sect. 12.1.3.2) using the nitrile hydratase from *Pseudomonas chlororaphis* B23. Starting at a substrate concentration of 1.5 M, high above the solubility level (0.45 M). The hydration was carried out in a two phase system. The nitrile hydratase showed outstanding stability at these high substrate concentrations. Sequential addition of the substrate, instead of starting at a high concentration, only slightly improved the stability. Increased stability could be achieved by the addition of butyric acid to the medium. However, the higher stability has to be traded off with a lower activity caused by the inhibition of the nitrile hydratase by butyrate (see Sect. 12.1.3.4).

12.1.3.4
Activation and Stabilization of Nitrile Hydratases

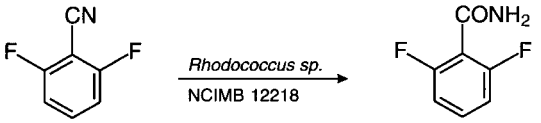
Iron-dependent nitrile hydratases, for example from *Rhodococcus* R312 or *Pseudomonas chlororaphis*, exhibit a remarkable dependency on light.

The enzymes, after being inactivated by aerobic incubation in the dark, regain their activity when exposed to light irradiation [12, 77]. Using different spectrophotometric techniques (ENDOR, EXAFS, FTIR, UV-VIS and X-ray) this phenomenon has been studied extensively in recent years. It has now been confirmed that the deactivation is caused by the reversible binding of nitric oxide to the non-heme iron center in a 1:1 stoichiometric complex. Upon irradiation the complex is destroyed and the activity of the nitrile hydratase is restored [14, 27, 78–81].

Another interesting characteristic of the iron-dependent nitrile hydratases is their stabilization during purification and storage by alcanoic acids such as butyric acid, hexanoic acid and valeric acid. The effect has already been described by Nagasawa et al. in 1987 [13]. However, only in recent years has the role of the acids been clarified by spectroscopic studies. Studying the EPR signals of the nitrile hydratase from *Brevibacterium* R312, Kopf et al. showed that butyric acid interacts with the iron in the active site of the nitrile hydratase, stabilizing the enzyme but, at the same time acting as a competitive inhibitor [82].

12.1.3.5
Nitrile Hydrolysis in Organic Solvents

Most nitrile bioconversions published have been conducted in aqueous media and consequently few data are available on the effect of solvents on enzymatic nitrile hydrolysis. Such studies seem highly justified in order to investigate the effects of different solvents or co-solvents on substrate specificity, conversion rate, stereoselectivity, and catalyst half-life.



Scheme 12.1-18.

De Raadt et al. reported on the inhibition of nitrile hydrolysis by various solvents [83].

However, production of 2,6-difluorobenzamide (Scheme 12.1-18) was effected in 99.5 % n-heptane using the nitrile hydratase from *Rhodococcus* sp. NCIMB 12218 [84]. The enzymatic reaction was found to be activated by light (see 12.1.3.4). More recently, Layh and Willetts have studied nitrile transformations in various organic solvents and biphasic mixtures using a nitrilase from *Pseudomonas* sp. DSM 11 387 and a nitrile hydratase from *Rhodococcus* sp. DSM 11 397 [85]. The enzymes exhibited good stabilities in biphasic mixtures with hydrophobic solvents when dispersed in

the buffer-saturated higher alcohols 1-hexanol, 1-heptanol, 1-octanol and 1- decanol, respectively. The nitrilase still retained 58%, 49%, 44% and 47% activity, while the nitrile hydratase only showed low activities (2–5%).

12.1.3.6
Large Scale Production of Acrylamide

Acrylamide monomer is an important chemical commodity produced on a multi-hundred thousand ton scale for the production of polymers and copolymers. The preferred manufacturing process is by the catalytic hydration of acrylonitrile at 70–120 °C using reduced Raney copper as the catalyst; the initial concentration of acrylonitrile being around 4 M. There are several shortcomings to this process, among which are the high level of acrylic acid formed and byproduct formation [2, 3].

An enzymatic acrylonitrile hydration was first patented in 1981 [86]. Many nitrile hydratases of different origin have been shown to be able to convert acrylonitrile into acrylamide. However, a major problem associated with biocatalysis for production of acrylonitrile is the short half-life of the enzyme due to substrate and product inhibition. Acrylonitrile is a strong alkylating agent which reacts by Michael addition with the sulfhydryl groups of proteins [68, 69].

The Mitsubishi Rayon Corp. (the former Nitto Chemical Industry Co.) established the industrial production of acrylamide in 1985 using immobilized cells of *Rhodococcus* sp. N-774 [3, 69, 74]. In 1988 a hyperproducing mutant strain of *Pseudomonas chlororaphis* B23 was chosen for production. As in *Rhodococcus* sp. N-774, the active

Table 12.1-2. Operating conditions for acrylamide production.

Reaction conditions	Productivity	
pH 7.5–8.5	Conversion acrylonitrile	> 99.9%
Temperature 0–5 °C	Yield of acrylamide	> 99.9%
Acrylonitrile concentration in the reactor 1.5–2.0%	Acrylamide concentration from the reactor	27–30%

Table 12.1-3. Comparison of enzyme data of three types of nitrile hydratases.

Parameter	*Rhodococcus* sp. N-774	*Pseudomonas chlororaphis* B23	*Rhodoccus rhodochrous* J1
Tolerance to acrylamide (%)	27	40	50
Acrylic acid formation	very little	barely detected	barely detected
Cultivation time (h)	48	45	72
Activity of culture broth (units mL^{-1})	900	1400	2100
Specific activity (units per mg cells)	60	85	76
Cell yield (g L^{-1})	15	17	28
Acrylamide productivity (g per g cells)	500	850	> 7000
Total amount of production (t per year)	4000	6000	30.000
Final concentration of acrylamide (%)	20	27	40
First year of production scale	1985	1988	1991

Table 12.1-4. Comaprison of enzyme data of three types of nitrile hydratases.

Parameter	*Rhodoccus* sp. N-774	*Pseudomonas chlororaphis* B23	*Rhodoccus rhodochrous* J1
Molecular mass	70.000	100.000	505.000
Subunit molecular mass	α 27.000 β 27.500	α 25.000 β 25.000	α 26.000 β 25.000
Metal	Fe^{III}	Fe^{III}	Co
Optimum temperature (°C)	35	20	35–40
Heat stability (°C)	30	20	50
Optimum pH	7.7	7.5	6.5
pH stability	7.0–8.5	6.0–7.5	6.0–8.5
Substrate specificity	aliphatic nitriles	aliphatic nitriles	aliphatic and aromatic nitriles
Activation by light irradiation	+	–	–
Formation type	constitutive	inducible (methacrylamide)	inducible (urea)

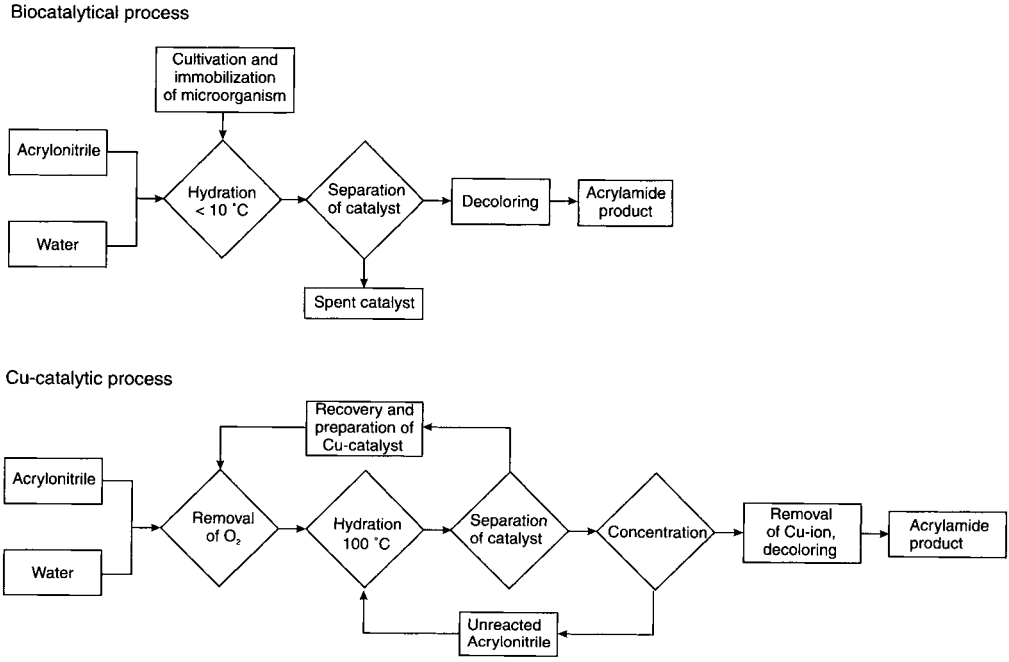


Figure 12.1-4. Comparison of the biocatalytic and the conventional chemical process for acrylamide production.

biocatalyst in *Pseudomonas chlororaphis* B23 is also a nitrile hydratase containing ferric ion as the cofactor[2, 74].

Current acrylamide production at Mitsubishi using bioconversion is around 40 000 tonnes per year. Using a highly improved cobalt-containing nitrile hydratase from *Rhodococcus rhodochrous* J1, final product concentrations of around 700 g L^{-1}

can be obtained[87]. The reaction is performed at 5–10 °C in order to reduce cell degradation and enzyme inhibition. No data have been published on the half-life of the *Rhodococcus rhodochrous* J1 nitrile hydratase under production conditions. A good summary of the biocatalytic production of acrylamide has been given by Yamada and co-workers[74, 87] (see Tables 12.1-2 to 12.1-4).

In Fig. 12.1-4 a comparison is presented of the enzymatic and the conventional chemical processes for acrylamide production.

12.1.4
Availability and Industrial Future of Nitrile Hydrolyzing Biocatalysts

Although nitrile-hydrolyzing enzymes have attracted considerable interest as promising "green catalysts", none of these enzymes are presently available as commercial products. Thus studies on nitrile biotransformations have been conducted with a variety of enzyme preparations ranging from resting cells, immobilized whole cells, cell-free extracts, immobilized enzymes and pure soluble enzymes. However, nowadays several nitrile hydratases and nitrilases have been cloned and overexpressed, giving rise to highly efficient and well defined catalysts[6, 7]. This not only provides commercial access to even more interesting catalysts, but also opens the way for the application of modern molecular biological methods for further optimization.

Within the recent years several industrial processes based on nitrile hydrolyzing enzymes have been introduced, as has been discussed above. This number is now expected to increase rapidly, due to the better availability of these biocatalysts.

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12.2
Formation and Hydrolysis of Amides

Birgit Schulze and Erik de Vroom

12.2.1
Introduction

Organic amides and acids are versatile precursors to the production of various commercial products; nowadays these compounds are mainly produced chemically. However, owing to environmental considerations and the increasing demand for chiral amides and acids there is a strong tendency to explore and develop biocatalytic production processes.

This section gives a global overview of the potential of microorganisms and enzymes to catalyze the regio- and enantioselective hydrolysis and formation of amides.

The biocatalytic hydrolysis of amides and also the enzyme catalyzed formation of amides and the synthesis of (semisynthetic) antibiotics is included in this section.

12.2.2
Enzymatic Formation of Amides

Currently two biocatalytic methods are known for the production of amides:

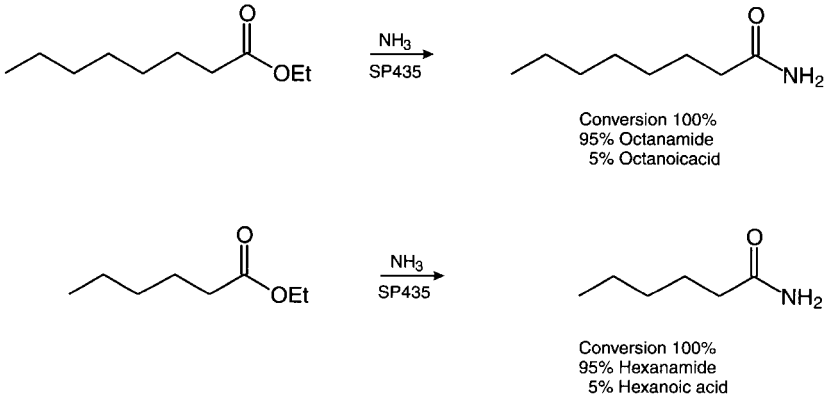
- The hydrolysis of nitriles using nitrile hydratases (for example: acrylamide production).
- The enzymatic ammoniolysis of carboxylic esters and amidation of carboxylic acids.

In the previous Section 12.1 the formation of amides from the corresponding nitriles is well addressed. The latter method, the enzyme catalyzed reaction of carboxylic esters or acids with ammonia or amines yielding amides, has only recently been studied in depth. Encouraging results have been described, especially in the field of amidation of esters, a technology now being used by BASF to produce optically pure amines (*vide infra*).

Lipases and esterases comprise a versatile group of enzymes that catalyze the hydrolysis of esters, esterifications and transesterifications via an acyl enzyme intermediate (Chapter 11). Various other nucleophiles can attack this acyl enzyme complex in addition to water.

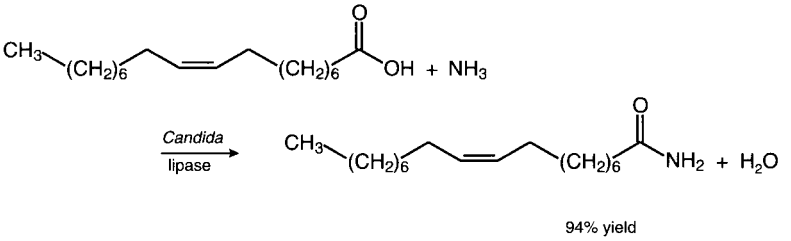
In recent years several authors have also shown that NH_3 and amines can act as nucleophiles leading to the formation of amides [1, 2].

Initially De Zoete et al. showed that fatty acid esters could be converted into the corresponding amides by bubbling gaseous NH_3 through the reaction mixture containing lipase B from *Candida antarctica* (SP 435 from Novo-Nordisk) in *tert*-butyl alcohol. As shown in Scheme 12.2-1 very good yields can be achieved. This enzyme has also been used in related studies by other authors. A conclusive summary can be found in [3].



Scheme 12.2-1.

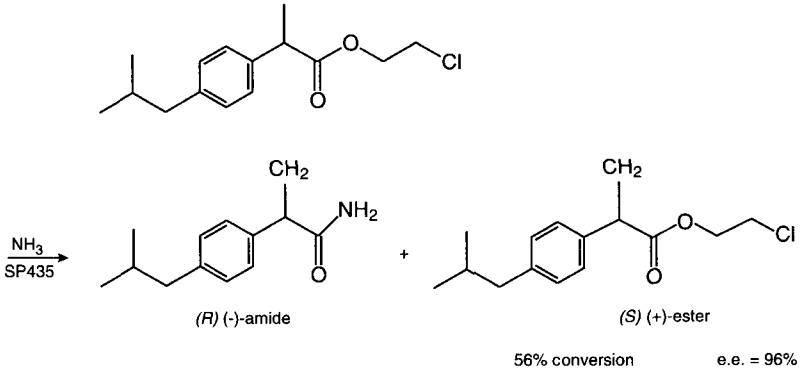
Further to this kinetic approach, the thermodynamic ammoniolysis has also been studied. Here the amide is formed directly by the reaction of a carboxylic acid with ammonia. Because these reactions are governed by the equilibrium concentrations of substrates and products, the solubility of the reactants is of major importance. The optimal conditions for an efficient ammoniolysis of butyric acid and oleic acid have now been reported. Oleamide, which has been reported to have a pharmacological application as a sleep inducing drug[4] has been derived from the acid by direct ammoniolysis in an efficient manner with good yields (Scheme 12.2-2)[5].



Scheme 12.2-2.

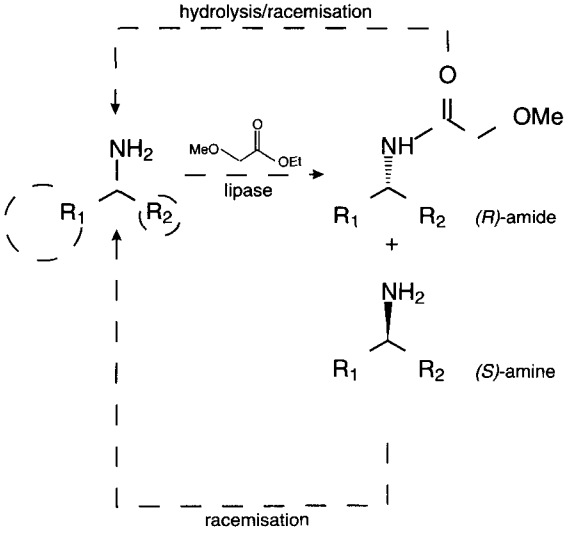
An interesting effect was found whilst studying the enantioselectivity of these reactions. Although several esters (ethyl-2-chloropropionate, ethyl lactate, ethyl-2-hydroxy hexanoate and ethyl-2-methyl butyrate) were converted into the amides with only low to moderate ee values, the ammoniolysis of ibuprofen (2'-chloroethyl)ester was highly enantioselective. At 56% conversion the ee of the remaining *(S)*-ester was 96% (Scheme 12.2-3), corresponding with an *E* value of the ammoniolysis of 28.

In comparison, the ester hydrolysis of ibuprofen (2'-chloroethyl)ester catalyzed by the same enzyme proceeded with an *E* value of only 3.5[2]. The same phenomenon has been observed in the ammoniolysis of 4-methyloctanoic acid. Here an *E* value of 76 was determined for ammoniolysis, whereas in the transesterification reaction an E-value of only 23 was found[5, 6].



Scheme 12.2-3.

It has now been reported that the amidation of esters will be used by BASF in the industrial production of amines. A broad range of amines become available in their optically pure form by kinetic resolution using a lipase from *Pseudomonas* sp. DSM 8246 in the amidation of methoxyacetic acid ethyl ester (see Scheme 12.2-4) [7].



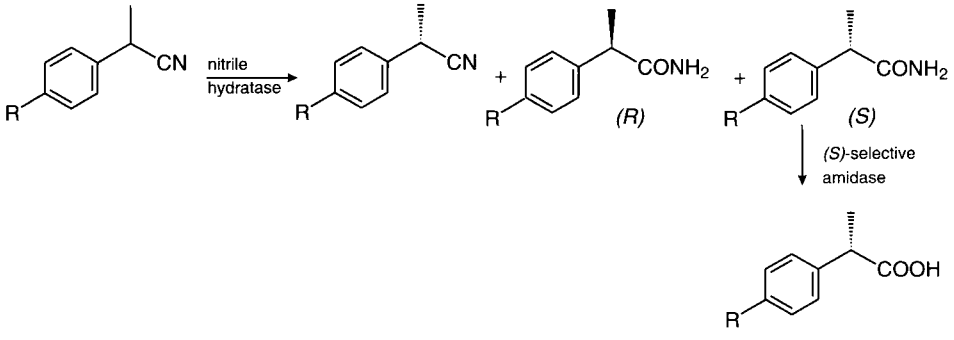
Scheme 12.2-4.

A comparitive study of a variety of lipases and lipase preparations in such alkoxycarbonylation reactions of amines has been presented by Sinisterra and co-workers [8].

12.2.3
Enzymatic Enantioselective Hydrolysis of Amides

12.2.3.1
Hydrolysis of Carboxylic Amides

Although amidase activities have been known for quite some time, it is only in recent years that the increasing demand for chiral drugs and herbicides has triggered their exploitation as biocatalysts to a great extent. Many, but not all, amidases have been identified in microorganisms also exhibiting nitrile hydratase activity. In some cases where the enantioselective transformation of the nitrile to the acid can be observed, the selectivity is based on the high selectivity of the amidases rather than on the discrimination by the nitrile hydratase. Thus, using these enzyme combinations, both the *(R)*-amides and the *(S)*-acids can be obtained by such a double enantiomeric selection. The first catalytic step is carried out by a nitrile hydratase with a slight preference for the *(R)*-enantiomer. At high conversions this will lead to a mixture of *(S)*-nitrile and *(R)*- and *(S)*-amide, the latter being subsequently hydrolyzed with high selectivity by an *(S)*-selective amidase to yield the *(S)*-acid[77,78] Scheme 12.2-5).

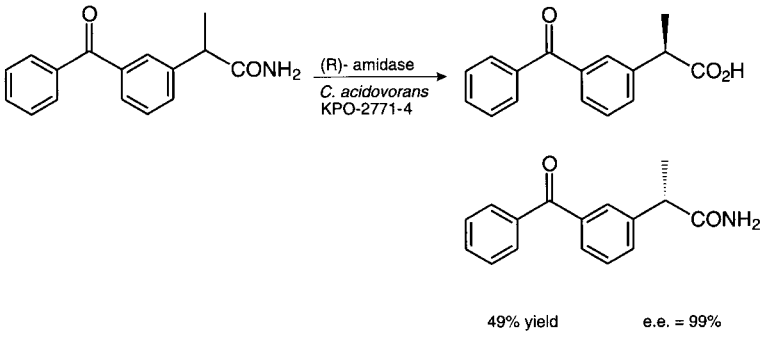


Scheme 12.2-5.

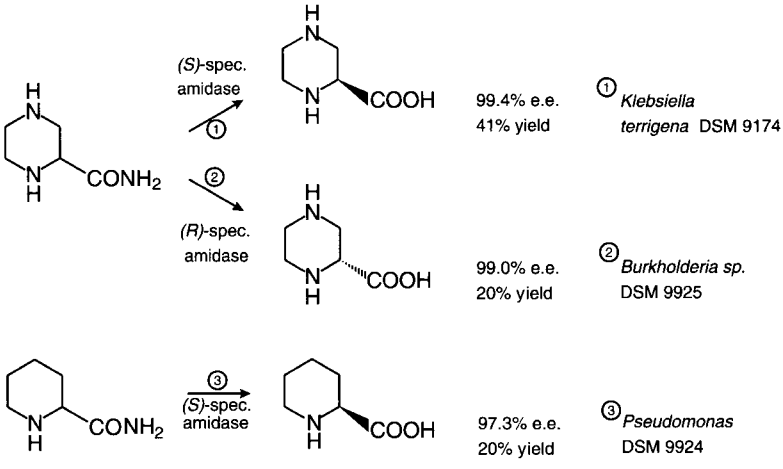
Other examples of *(S)*-selective amidases are described for the production of *(S)*-2-(4'-chlorophenyl)-3-methyl butyric acid[9], *(S)*-ibuprofen[10], *(S)*-naproxen[11] and L-carnitine[12, 13].

In addition to the more common *(S)*-amidase activities, *(R)*-specific enzymes have also been identified. Thus *(S)*-ketoprofenamide has been derived from the racemic mixture using a biocatalyst from *Comamonas acidovorans* KPO 2771–4 to hydrolyze the *(R)*-enantiomer with high selectivity[14] (see Scheme 12.2-6).

Lonza AG has reported on the use of enantioselective amidases for the resolution of piperazine-2-carboxamide and piperidine-2-carboxamide using whole cell biocatalysts from *Klebsiella terrigena*, *Pseudomonas fluorescence* and *Burkholderia* sp., the last containing an *(R)*-selective amidase (Scheme 12.2-7)[15]. Furthermore, several amidases exhibiting high selectivities [either *(S)*- or *(R)*-] towards 2-arylpropiona-



Scheme 12.2-6.



Scheme 12.2-7.

mides have been identified. A good overview has been given by Wieser and Nagasawa [79].

In recent years the availability of several amidases has been improved by cloning and overexpression [16–19] resulting in biocatalysts of high activities which can readily be used for industrial purposes. Furthermore, homology studies have been carried out to identify the common features of this class of enzymes [20].

12.2.3.2
Hydrolysis of Amino Acid Amides

The conversion of amino acid amides into chiral amino acids has been the subject of a large number of monographs and reviews [21–29]. In this section information will be given on amidases and aminopeptidases that have been reported for the stereoselective hydrolysis of amino acid amides.

12.2.3.2.1 Production of Chiral α-H-α-Amino Acids

At DSM a very efficient and universally applicable industrial process for the production of both optically pure L- and D-amino acids has been commercialized[25, 27]. Pivotal in this process is the enantioselective hydrolysis of D,L-amino acid amides. The stable D,L-amino acid amides are prepared efficiently under mild reaction conditions starting from simple raw materials (Fig. 12.2-1). The reaction of an aldehyde with hydrogen cyanide in ammonia (Strecker reaction) affords the amino nitrile. The amino nitrile is converted in a high yield into the D,L-amino acid amide under alkaline conditions in the presence of catalytic amounts of acetone. The

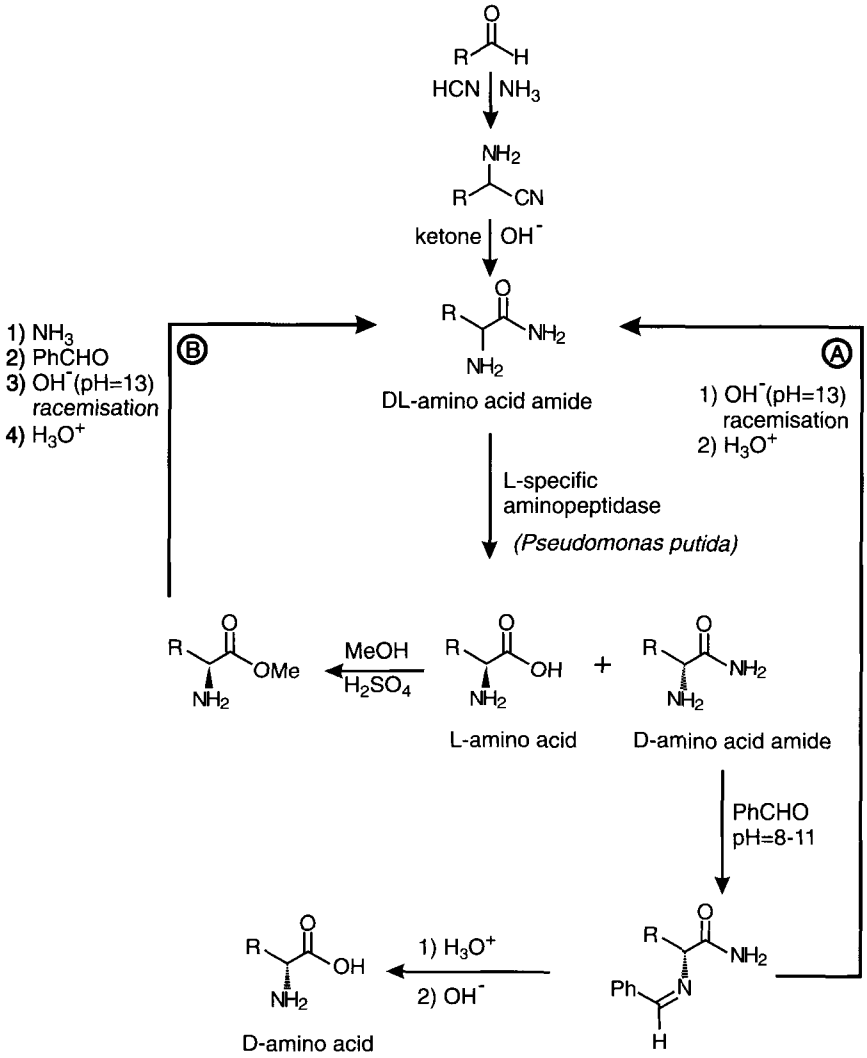
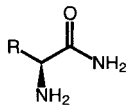


Figure 12.2-1. DSM's chemo-enzymatic route for the production of chiral α-H-amino acids.

Table 12.2-1. Substrates by *Pseudomonas putida* cells.



R	R	R	R
H_3C-		—CH_2—	H_3CS-CH_2-CH_2-
H_3C-CH_2-	Cl	HO— —CH_2	H_3CS-CH_2-CH_2-CH_2-
$(H_3C)_2CH$-	HO—	—CH_2—CH_2—	H_2C=CH-CH_2-
$(H_3C)_2CH$-CH_2-		S	Fe

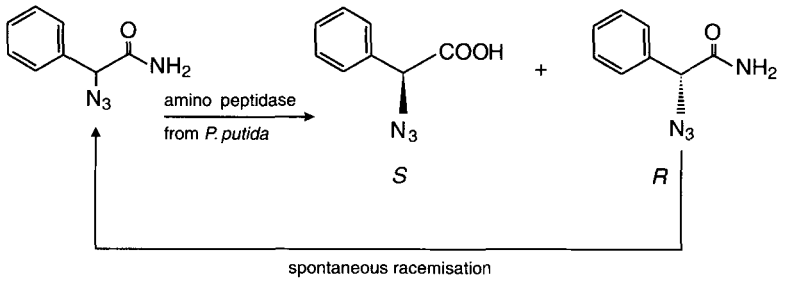
resolution step is accomplished with permeabilized whole cells of *Pseudomonas putida* ATCC 12633; a stereoselectivity of nearly 100% ($E > 100$) on hydrolyzing only the L-amino acid amide is combined with a very relaxed substrate specificity (see Table 12.2-1)[27, 29–31].

Not only the smallest optically active amino acid, for example alanine, but also valine, leucine, several (substituted) aromatic amino acids, heterosubstituted amino acids (methionine, homomethionine and thienylglycine) and even an imino acid, proline, are obtainable in both the L- and the D-form. Furthermore, this biocatalyst has recently been reported to hydrolyze azido amino acid amides with high enantioselectivities as well (*vide infra*)[32].

No enzymatic side effects are observed and substrate concentrations up to 20% by mass can be used without affecting the enzyme activity. The biocatalyst is active in a broad pH-range and can be used in soluble form in a batchwise process; thus poorly soluble amino acids can be resolved without technical difficulties.

Re-use of the biocatalyst is possible. A very simple and elegant alternative to the use of ion-exchange columns or extraction to separate the mixture of D-amino acid amide and the L-amino acid has been elaborated at DSM. Thus addition of one equivalent of benzaldehyde (with respect to the D-amino acid amide) to the enzymatic hydrolysate results surprisingly in the formation of a water insoluble Schiff base with the D-amino acid amide which can be easily separated. Acid hydrolysis (H_2SO_4' HHal, HNO_3 etc.) results in the formation of the D-amino acid amide, which can be hydrolyzed by cell-preparations *of Rhodococcus erythropolis*, yielding the D-amino acid. The amidase from this organism lacks stereoselectivity. This option is very useful for amino acids that are highly soluble in the neutralized reaction mixtures obtained after acid hydrolysis of the amide.

Process economics dictate the recycling of the unwanted isomer. Path A in Fig. 12.2-1 illustrates that racemization of the D-*N*-benzylidene amino acid amide is facile and can be carried out under very mild reaction conditions. After removal of



Scheme 12.2-8.

the benzaldehyde the D,L-amino acid amide can be recycled. This option means that 100% conversion into the L-amino acid is theoretically possible. A suitable method for racemization and recycling of the L-amino acid (path B, Fig. 12.2-1) comprises the conversion of the L-amino acid into the ester in the presence of concentrated acid, followed by addition of ammonia, resulting in the formation of the amide. Addition of benzaldehyde and racemization by base (pH 13) gives the D,L-amino acid amide. In this way 100% conversion into the D-amino acid is possible. For the production of 2-azidophenylacetic acid an even more elegant way of achieving 100% yield of one enantiomer has been reported. Under the conditions used for the resolution a spontaneous racemization of the substrate is achieved, resulting in a dynamic kinetic resolution with a theoretical yield of 100% (Scheme 12.2-8). The distinct advantages of the aminopeptidase process are:

- The substrate for the enzymatic hydrolysis is a precursor of the amino acid; the number of chemical steps can be kept to a minimum.
- The use of relatively cheap whole cell biocatalysts contributes to the economical feasibility of the procedure.
- Both L- and D-amino acids can be prepared with a very high optical purity.

The aminopeptidase from *Pseudomonas putida* ATCC 12633 has also recently been cloned and overexpressed in *E. coli* resulting in a highly efficient whole-cell biocatalyst for industrial applications [29]. The specific activity of this new biocatalyst is substantially increased (25 times) compared with the specific activity of the *P. putida* wild type cells without changing the other positive characteristics of the aminopeptidase. Even though the aminopeptidase from *Pseudomonas putida* exhibits the relaxed substrate specificity described above, an α-hydrogen atom in the substrate is an essential structural feature for the enzymatic activity. Therefore this enzyme can not be used for the resolution of higher substituted amino acids.

Recently, a new biocatalyst with a broad substrate spectrum of L-specific amidase activity has been identified at DSM. Of 125 microorganisms that were able to use α-hydroxy acid amides as the sole nitrogen source, *Ochrobactrum anthropi* NCIMB 40321 was selected for its ability to hydrolyze racemic amides with high L-selectivity. The substrate specificity of whole *Ochrobactrum anthropi* cells is remarkably wide and ranges from α-H-α-amino, α-alkyl-α-amino, and *N*-hydroxy-α-amino acid amides to α-hydroxy acid amides [33]. After 50% conversion, both the L-acids formed and the

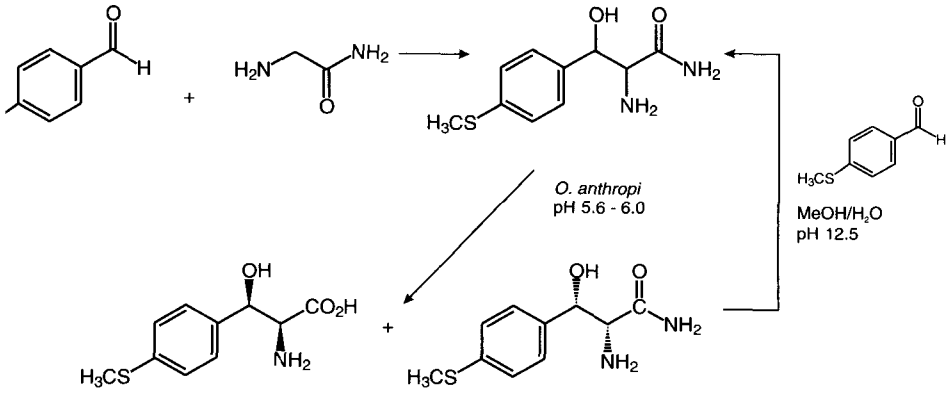
Table 12.2-2. Substrate specificty of *Ochrobactrum anthropi* cells. L-selective hydrolysis[a] of amides by *Ochrobactrum anthropi*.

Substrate	Relative activity (%)
D,L-α-valine amide	25
D,L-α-methylamide	5
D,L-α-methylleucine amide	15
D,L-*tert* leucine amide	1
D,L-α-cinnamylalanine amide	17
D,L-phenylglycine amide	100[b]
D,L-α-methylphenylglycine amide	2
D,L-α-ethylphenylglycine amide	4
D,L-α-propylphenylglycine amide	1
D,L-α-allylphenylglycine amide	4
D,L-α-benzylphenylglycine amide	0
D,L-*N*-hydroxyphenylglycine amide[c]	25
D,L-mandelic acid amide (MAA)	5

a Activities were measured at pH 8.0 (100 m*M* phosphate buffer) and 40 °C using 3.0 g L^{-1} of amide.
b A relative activity of 100 corresponds to 2000 nMol min^{-1} (mg dry mass)$^{-1}$. c Incubation performed under anaerobic conditions by flushing with nitrogen.

residual D-amides were present in > 99% enantiomeric excess and ammonia accumulated in stoichiometric amounts. The substrate specificity is illustrated in Table 12.2-2. Using this biocatalyst a new route to thiamphenicol, a synthetic analog to the antibiotic chloramphenicol has been developed (Scheme 12.2-9). A precursor of the biologically active *(1R,2R)*-enantiomer, the *(2S,3R)-para*-substituted 3-phenylserine is obtained by the enzymatic resolution. The residual enantiomer can be efficiently recycled via separation by Schiff base formation with the corresponding *para*-substituted benzaldehyde and subsequent transformation into the racemic *threo*-amides [34].

A D-aminopeptidase has been identified at the Sagami Research Institute by Asano et al. [35]. The group was successful, by using an enrichment culture technique, in selecting a microorganism (*Ochrobactrium anthropi* SCRC Cl-38) with D-aminopepti-



Scheme 12.2-9.

dase activity from a soil sample. The enzyme, which hydrolyzes D-alanine amide, was purified about 2800 fold. The molecular mass of the native enzyme was approximately 122000 Da, with two identical subunits having a molecular mass of about 59000 Da each. Remarkably, D-valine amide is hydrolyzed very slowly. Generally, the enzyme has higher affinity towards peptide substrates than towards amino acid amides. It does not act on peptides bearing an L-amino acid at the NH_2-terminus. Thus it exhibits a mode of action typical of aminopeptidases. The optimal pH for activity was 8.0. The immobilized enzyme was also active in organic solvents (benzene, butyl acetate, 1,1,1-trichloroethane)[36]. D-Alanine-(3-aminopentyl) amide was quantitatively synthesized in an amination reaction from D-alanine methylester and 3-aminopentane within 1 h.

Asano et al. have also purified a D-stereospecific amino acid amidase from another *Ochrobactrum anthropi* isolate[37, 38]. Recently, a new amidase from *Comamonas acidovorans* has been reported that exhibits a broad substrate specificity and also D-amino acid amidase activity[39]. In addition, a D-specific amidase has been identified in *Arthrobacter* sp. NJ-26[40]. In contrast to the D-selective enzymes of *Ochrobactrum* sp. and *Comomonas acidovorans*, the D-amide hydrolase identified in *Arthrobacter* sp. NJ-26 was very substrate specific: a good hydrolysis rate was only observed for D-alanine amide.

12.2.3.2.2 Synthesis of α-Alkyl-α-Amino Acids

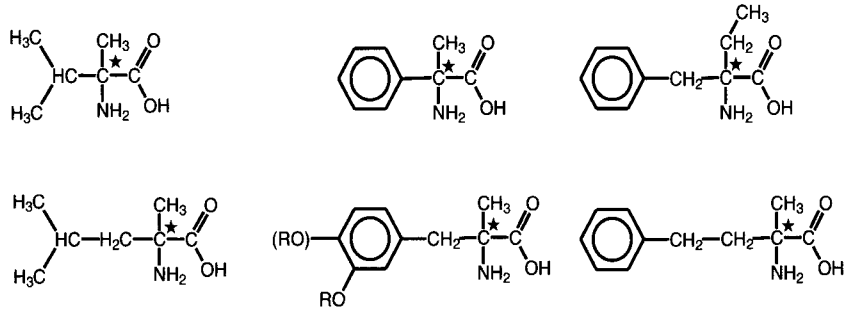
Within the pharmaceutical industry α-alkyl-α-amino acids are regarded as valuable building blocks. An example of this class is L-α-methyl-3,4-dihydroxyphenylalanine (L-methyl-Dopa), which is used as a drug to treat patients suffering from high blood pressure. More recently, medicinal chemists became interested in bio-active peptides containing α-alkyl-α-amino acids since they tend to freeze specific conformations and slow down enzymatic degradations dramatically[41]. Nowadays, many α-alkyl-α-amino acids have been found in nature. For example, L-isovaline is found in peptaibol antibiotics. Their influence on the conformational behavior of peptides is presently under active investigation. Several routes to enantiomerically pure α-alkyl-α-amino acids have been elaborated in recent years[26–28, 42, 43]. At DSM a *Mycobacterium neoaurum* biocatalyst has been obtained in a screening, which hydrolyzes a broad range of α-alkyl-α-amino acid amides with high enantioselectivities (Table 12.2-3).

The basis of the process leading to the enantiomerically pure acid is essentially the same as that for α-H-α-amino acids. However, in this case, a ketone is used as the starting material which undergoes a Strecker reaction, followed by hydrolysis of the resulting aminonitrile to form the racemic α-alkyl-α-amino acid amide. Enzymatic hydrolysis results in the formation of the L-α-alkyl-α-amino acid (Fig. 12.2-2).

Some characteristics of the process are:

- Using this process both L- and D-α-alkyl-α-amino acids can be produced.
- Permeabilized whole cells of *Mycobacterium neoaurum* ATCC 25795 or crude enzyme preparations can be used.

Table 12.2-3. Substrate specificity of the amidase activity of *Mycobacterium neoaurum* cells.

Products formed


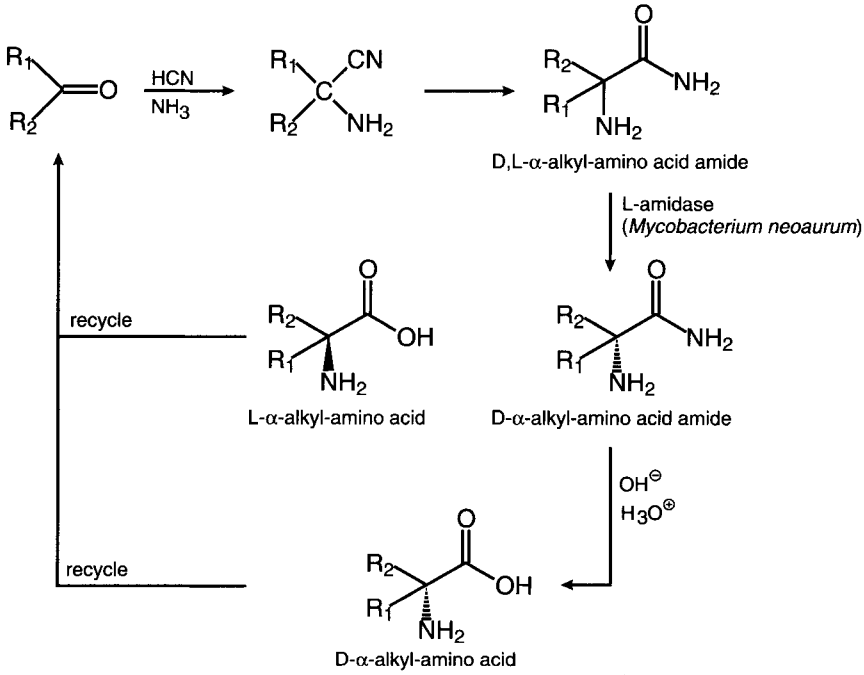


Figure 12.2-2. DSM's chemo-enzymatic route for the preparation of α-alkyl-α-amino acids.

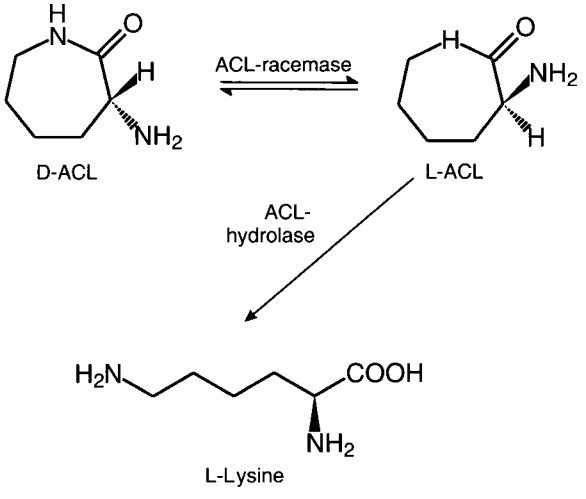
- Very high stereoselectivity (> 98 % ee) and a remarkably relaxed substrate specificity are observed (table 12-7).
- The enzyme is active in the pH range from 6.5 to 11, with a broad optimum from pH 8.0–9.5

Recently the enzyme has been purified and thoroughly characterized. It was identified as an amino acid amidase, most probably belonging to the group of

metallocystein hydrolases[29]. In addition to DSM, Ube company reported an analogous biocatalytic route to α-methyl phenylalanine. A *Pseudomonas fluorescens* (IFO 3081) showed the highest conversion (94%) but the stereoselectivity was relatively low (ee 93.4%)[44].

12.2.3.3
Hydrolysis of Cyclic Amides

Cyclic amides can also be hydrolyzed in a highly selective fashion using enzymes. A well known example in this respect is the biocatalytic production of L-lysine from D,L-α-amino-ε-caprolactam (D,L-ACL)[45–47]. This process is based on the combination of two enzymatic reactions: the enzymatic enantiospecific hydrolysis of L-α-amino-ε-caprolactam to L-lysine and the simultaneous racemization of the residual D-α-amino-ε-caprolactam (Scheme 12.2-10).



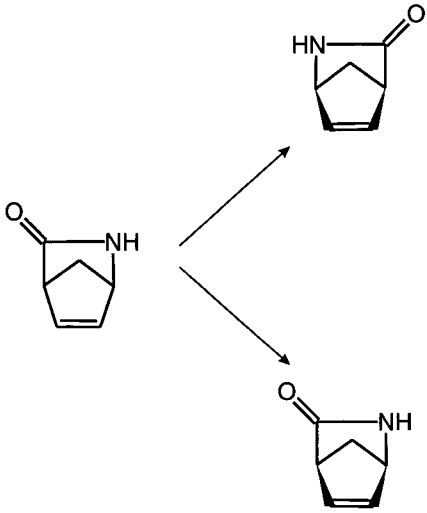
Scheme 12.2-10.

In this way L-lysine is produced from D,L-α-amino-ε-caprolactam, with a yield of almost 100%, by incubating the racemate with microbial cells *of Cryptococcus laurentti*, which possess L-α-amino-ε-caprolactamase activity, together with cells of *Achromobacter obae*, which possess α-amino-ε-caprolactam racemase activity.

The enzymatic hydrolysis of other cyclic amides was also investigated in order to obtain chiral precursors for antibiotics and/or HIV inhibitors. Using isolates capable of growth on a range of *N*-acyl compounds as the sole carbon and energy source, two strains were selected for the enantioselective hydrolysis of (±)-2-azabicyclo-[2.2.1]hept-5-en-3-one (Scheme 12.2-11). *Rhococcus equi* NCIMB 40231 selectively hydrolyzed the (-)-enantiomer, yielding (+)-lactam with > 98% ee (45% yield), whereas, *Pseudomonas solanacearum* NCIMB 40249 hydrolyzed the opposite enantiomer with great selectivity yielding (-)-lactam with > 98% ee (45%yield)[48, 49]. In

these biotransformations the relatively low concentration of enzyme (6 g dry mass L^{-1}), the high concentration of substrate (50 g L^{-1}), and the speed of the reaction (3 h) are worth noting. Moreover, mutant strains have been constructed which hyper-express the amidase activity. Subsequently it was shown that *Rhodococcus equi* cells can also be applied for the enantioselective hydrolysis of 6-azabicyclo[3.2.0]hept-3-en-7-one, yielding a precursor for the antifungal agent cispentacin[50].

Evidently, the use of the whole cell biocatalyst enables an efficient biotransformation with high substrate concentrations.



Scheme 12.2-11.

Recently, BASF has described the enantioselective hydrolysis of substituted lactams. Using strains of *Pseudomonas aeruginosa* and *Rhodococcus erythropolis* obtained from soil samples, both enantiomers of 5-vinylpyrrolidinon can be derived[51].

12.2.4
Selective Cleavage of the C-Terminal Amide Bond

In peptide synthesis selective deprotection of *C*- or *N*-terminal groups is common in most methods of chain elongation. The amide groups offer some advantages for *C*-terminal protection (enhanced chemical stability and increased solubility in water).

However, selective cleavage of this amide bond in the *C*-terminal position was previously impossible. Both chemical and biochemical methods also led to internal peptide bond hydrolysis, giving rise to difficult separation problems. Consequently the amide group has been rather unattractive for *C*-terminal protection in peptide synthesis.

Now, however this situation has changed. Steinke and Kula have isolated an unusual peptide amidase from orange flavedo, which is very selective for the

hydrolysis of the *C*-terminal amide bond of peptides[52]. The peptide amidase is free of any proteolytic activity, which would either hydrolyze internal peptide bonds of substrate peptides or side chain amide bonds.

The substrate spectrum of this enzyme includes protected and unprotected peptide amides and *N*-protected amino acid amides[53]. The chain length of the substrate peptide amide, as well as the amino acid composition, including the *C*-terminal amino acid side chain, are of minor importance. The amidase activity is stereoselective with regard to the *C*-terminal position, only L-amino acid amides are accepted. Unprotected amino acid amides are not hydrolyzed by this novel enzyme. The broad application of this enzyme is further extended by its broad pH activity range, from 6.5 to 9. Evidently, a new and useful biocatalyst is now available for selective deamination[54]. Recently the same group has also shown that the reverse reaction, the ammoniolysis of peptides, is catalyzed by this enzyme. Reducing the water activity by carrying out the reaction in acetonitrile containing 5% of water, Z-Gly-Phe-NH_2 has been derived in a thermodynamic ammoniolysis in yields of up to 35%[55].

12.2.5
Amidase Catalyzed Hydrolytic and Synthetic Processes in the Production of Semi-synthetic Antibiotics

Since the discovery of the β-lactam antibiotic penicillin G (Fig. 12.2-3) by Fleming in 1929, the use of antibiotics against pathogenic bacteria has increased dramatically. Penicillin G was initially used, which must be applied intravenously because of its instability in the stomach, but now penicillin V, which can be administered orally, has been introduced. However, as a result of the increasing resistance of bacteria, new antibiotics had to be developed. The semi-synthetic antibiotics, which often possess a broad spectrum of antibacterial activity, were produced by altering the side chain of penicillin G through acylation of the amine function of 6-aminopenicillanic acid (6-APA)[56].

The first commercial semi-synthetic antibiotic was ampicillin, which was introduced by Beecham in 1961[57]. A few years later a new class of antibiotics, the cephalosporins, was marketed. Some of the semi-synthetic cephalosporins are prepared from 7-aminocephalosporanic acid (7-ACA), others from 7-aminodesacetoxycephalosporanic acid (7-ADCA). 7-ACA is an intermediate that can be obtained from the fermentation product cephalosporin C; 7-ADCA is an intermediate that was discovered by Morin et al.[58] using chemical ring expansion of the penicillin nucleus (Fig. 12.2-4). The only difference between the two molecules is the absence of an acetoxy moiety in 7-ADCA. Today, the main intermediates for semi-synthetic cephalosporins (SSCs) and penicillins (SSPs), 7-ADCA and 6-APA, respectively, are produced in quantities of many thousands of tons annually in biocatalytic processes using penicillin amidases.

The coupling of the side chains to 6-APA and 7-ADCA is still performed chemically. However, in order to obtain improved coupling yields and to overcome the use of toxic and hazardous chemicals and solvents, several leading producers are

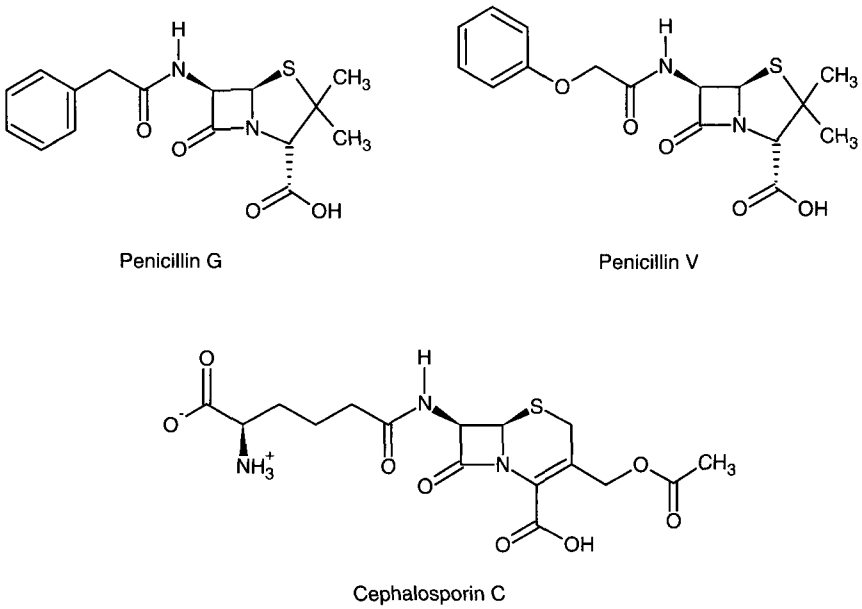


Figure 12.2-3. The three main fermentatively derived β-lactams.

currently investigating so-called "green routes" again utilizing penicillin amidases to perform this coupling reaction enzymatically.

12.2.5.1

Enzymatic Production of 6-APA, 7-ADCA and 7-ACA Using Amidases: Hydrolytic Processes

Owing to the increasing importance of semi-synthetic antibiotics, commercially feasible routes are of the utmost importance and several methods have been developed.

About a decade ago 6-APA and 7-ADCA were mainly produced by chemical deacylation of penicillin G, penicillin V or phenylacetyl 7-ADCA, the last of which was derived from chemical ring expansion of oxidized penicillin G. As a result of the fact that these processes were rather complex and employed hazardous reagents, for example pyridine, phosphorus pentachloride, nitrosyl chloride and dichloromethane, alternative processes have been developed. Penicillin amidases (E. C. 3.5.1.11) catalyze the hydrolysis of the linear amide bond in penicillin molecules producing both the β-lactam nucleus, 6-APA and the corresponding side chain without affecting the β-lactam amide bond in the four-membered ring. Based on their substrate specificity the penicillin amidases are grouped into three classes [59]:

1. Amidases that preferentially hydrolyze penicillin V (phenoxymethyl penicillin).
2. Amidases which are primarily active against penicillin G.
3. Amidases which are most active using ampicillin as the substrate.

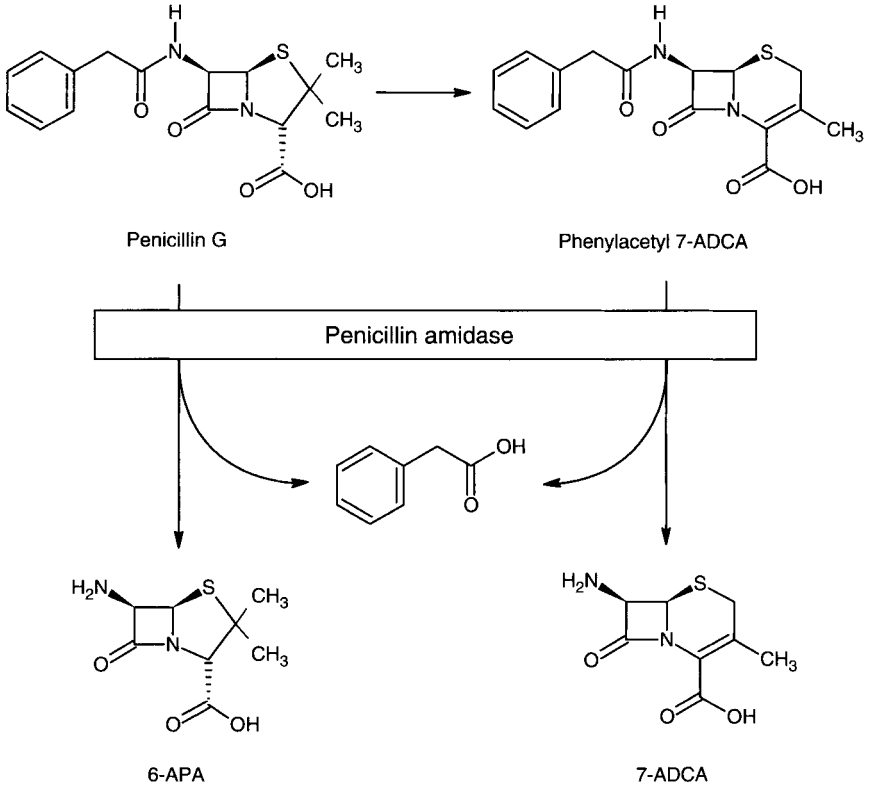


Figure 12.2-4. Enzymatic production of 6-APA and 7-ADCA. 6-APA is produced from penicillin G (or V) using the enzyme penicillin amidase. For the production of 7-ADCA, penicillin G is transformed chemically into phenylacetyl 7-ADCA. This transformation involves oxidation of penicillin G followed by a ring expansion reaction in which a molecule of water is excluded. Again, penicillin amidase is used for the hydrolysis of phenylacetyl 7-ADCA into 7-ADCA. Both the production of 6-APA and of 7-ADCA involve the liberation of phenylacetic acid, a molecule that can be recycled into the fermentation process.

Penicillin G amidases, in contrast to penicillin V amidases, display a fairly relaxed substrate specificity. Consequently penicillin G amidases can also be used for various other applications[60, 61].

Major breakthroughs that facilitated enzyme application on an industrial scale were improvements in the area of enzyme isolation, purification and immobilization. Thus, the development of genetically engineered microorganisms accounted for the high yield production of penicillin amidases. Also, the introduction of immobilized enzyme systems, both for whole cell systems and for the isolated and purified amidases[59, 62, 63], resulted in prolonged enzyme stability enabling reuse and continuous process modes. As a result of this, the enzymatic routes currently display far better economics for both 6-APA and 7-ADCA production (Fig. 12.2-4) compared with their chemical counterparts.

Nowadays, excellent penicillin amidases from various sources are being used on

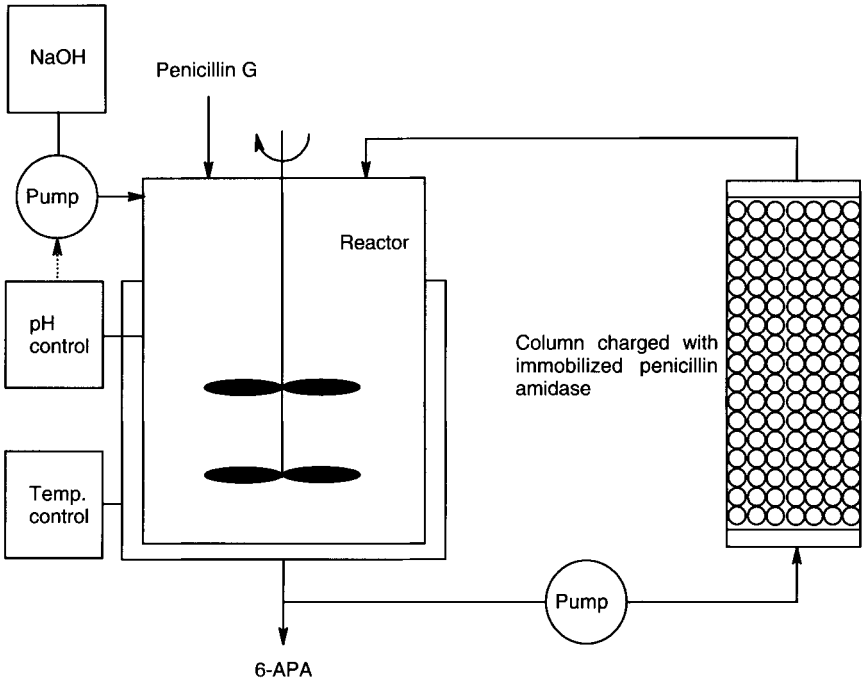


Figure 12.2-5. Schematic representation of industrial production of 6-APA using column packed immobilized penicillin amidase.

an industrial scale for producing either 6-APA or 7-ADCA[59, 63]. These biocatalytic processes are generally performed batchwise at 35–40 °C and pH 7.5–8.5[62, 64,65]. Upon formation of 6-APA and 7-ADCA the side chain acid is liberated, which causes a drop in the pH of the reaction mixture. This pH change results in a decrease in the reaction velocity. Since a higher starting pH is not desired because of enzyme deactivation and β-lactam ring hydrolysis, a strict control of the pH is necessary during the process. Generally, pH adjustment occurs separately from the immobilized enzyme, either by packing the immobilized enzyme in columns, as outlined in Fig. 12.2-5, or by cycling the contents of the enzyme reactor through a sieve, retaining the immobilized biocatalyst over a small pH control vessel. Currently, immobilized penicillin amidases can be reused up to 600 times[59]. After completion of the hydrolytic reaction the immobilized biocatalyst is separated from the liquid and the products 6-APA or 7-ADCA are precipitated at their iso-electric points and collected by filtration. After washing and drying an extremly pure product is obtained[65].

In addition to 7-ADCA, 7-ACA is also a very useful intermediate for the production of other SSCs (for example cefazolin, cefotaxime, ceftriaxone and cefuroxime). Until recently, 7-ACA was produced chemically from cephalosporin C using the phosphorus pentachloride process or the "Delft Cleavage"[65]. As a result of the good experiences with penicillin amidases and the increasing concern about the amount

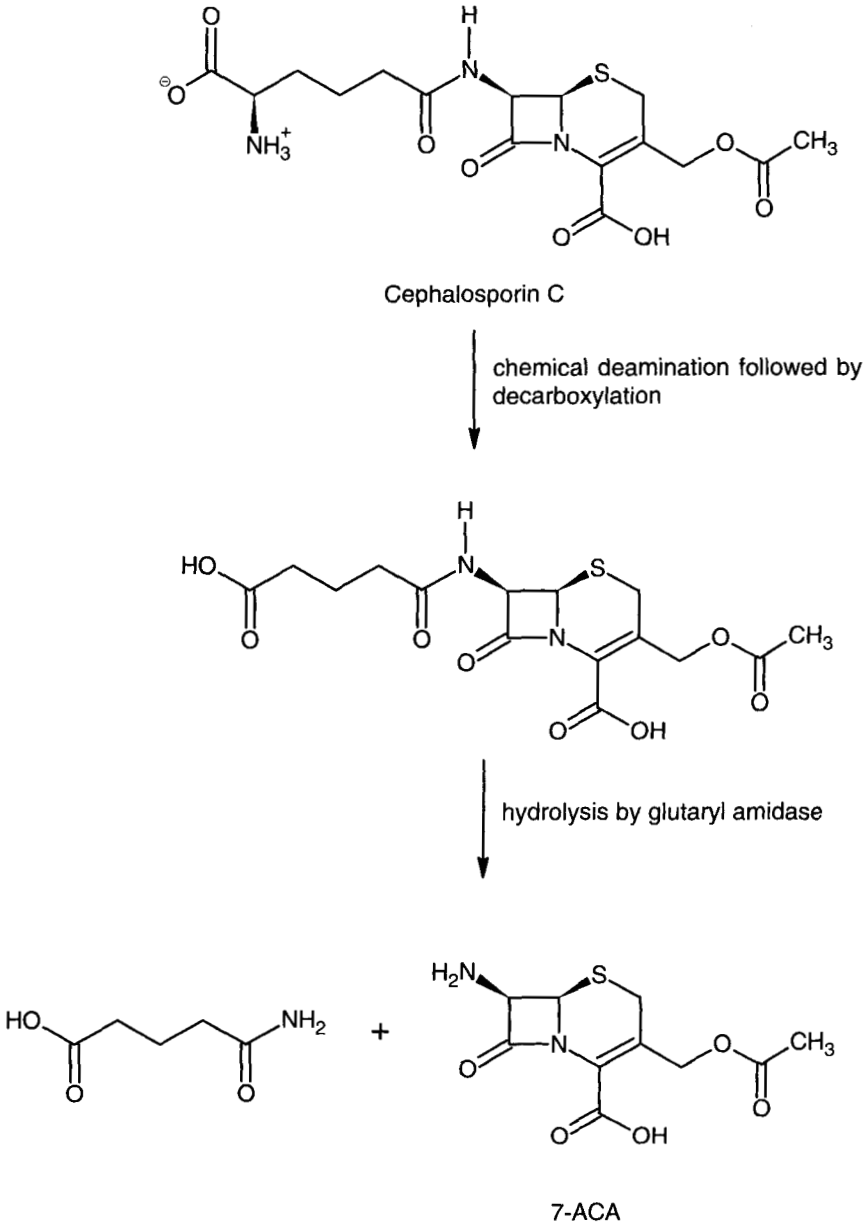


Figure 12.2-6. Chemo-enzymatic production of 7-ACA from cephalosporin C.

of waste being produced in chemical side chain cleavage processes, several companies are engaged in the development of enzymatic processes for the production of 7-ACA. Several years ago Asahi commercialized a chemo-enzymatic process in which cephalosporin C is oxidatively deaminated to glutaryl-7-ACA, which is

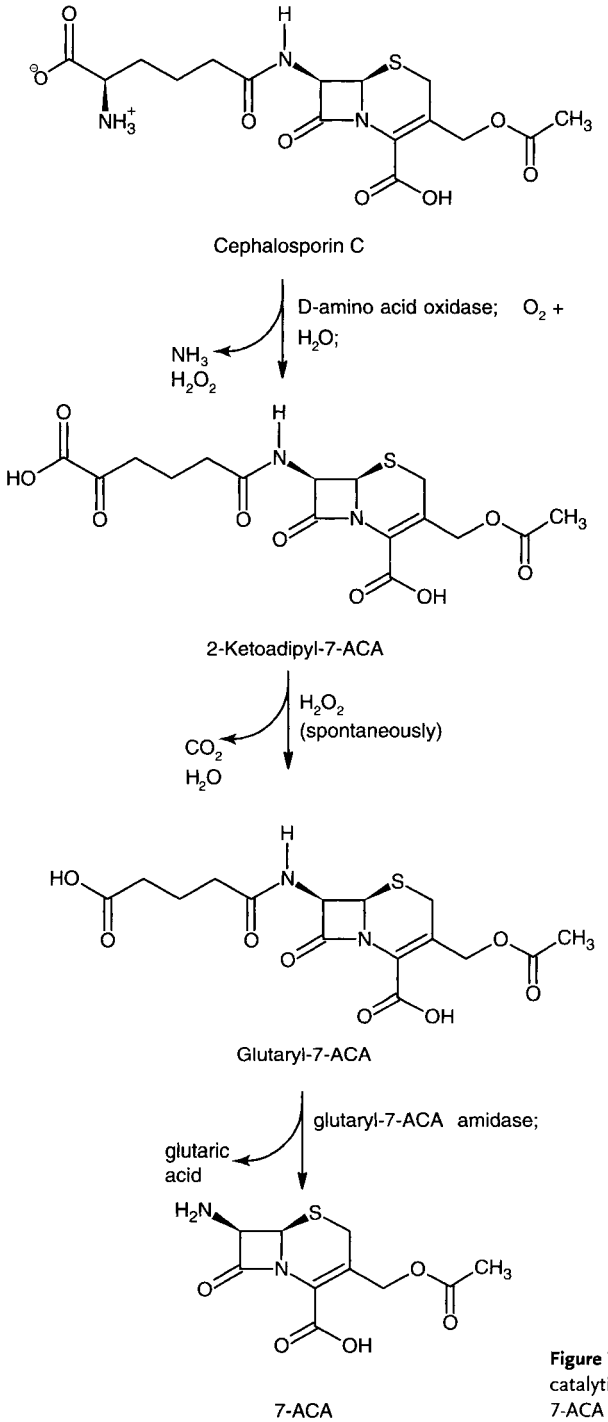


Figure 12.2-7. Two-enzyme biocatalytic process for production of 7-ACA from cephalosporin C.

subsequently hydrolyzed enzymatically using a glutaryl amidase from a *Pseudomonas* species (Fig. 12.2-6)[65]. Currently, the first step of this process is also carried out enzymatically. Using a D-amino acid oxidase cephalosporin C is oxidized to the corresponding α-ketoadipyl derivative. This latter compound spontaneously decarboxylates to give glutaryl-7-ACA (Fig. 12.2-7)[65, 67].

So far no direct deacylation of cephalosporin C has been commercialized, although enzymes have been identified that can indeed catalyze this one-step hydrolysis[68]. After optimization of the production of this biocatalyst, and possibly improvement of its intrinsic properties, it is very likely that a one-step enzymatic hydrolysis of cephalosporin C will be industrialized.

12.2.5.2
A New Fermentation-based Biocatalytic Process for 7-ADCA

With the aid of metabolic pathway engineering a large step forward has now been realized in the production of 7-ADCA by adapting processes within penicillin producing organisms. Thus, the conversion of the five-membered penicillin ring into the six-membered cephalosporin ring can now be performed within the microorganism as outlined in Fig. 12.2-8. By modifying the responsible gene, the penicillin producing mould can be set to produce a 7-ADCA derivative directly. Thus, several chemical steps from penicillin via penicillin oxide to 7-ADCA can be omitted[69].

Because of a newly introduced gene, the substrate specificity of the engineered strain changed. Now, dicarboxylic acid is used as the externally added side chain, instead of phenylacetic acid as in penicillin G.

Later in the process this side chain is removed enzymatically, using an enzyme quite similar to the glutaryl amidase from *Pseudomonas* sp. as in the enzymatic production of 7-ACA. For the production of 7-ADCA and the dicarboxylic acid amidase, new plants are currently under construction at DSM in The Netherlands. Compared with the old process for the production of 7-ADCA, the major advantages of this process are higher purity of the end product, much greater energy efficiency and almost complete absence of organic solvents.

12.2.5.3
Enzymatic Formation of Semi-synthetic Antibiotics: Synthetic Processes

The chemical synthesis of semi-synthetic antibiotics (SSAs) from a β-lactam nucleus (such as 6-APA, 7-ACA, or 7-ADCA) and a side chain (such as D-(-)-phenylglycine or an aminothiazoleiminoacetic acid derivative) is difficult to carry out in a single step since both reactants have functional groups that can easily form undesired covalent bonds.

In order to obtain the desired product in high yield it is necessary to activate the carboxyl function of the acylating agent, to temporarily protect interfering amino functions, to effect the formation of the amide bond and to remove the protecting groups. Moreover, this condensation should be performed under conditions that will

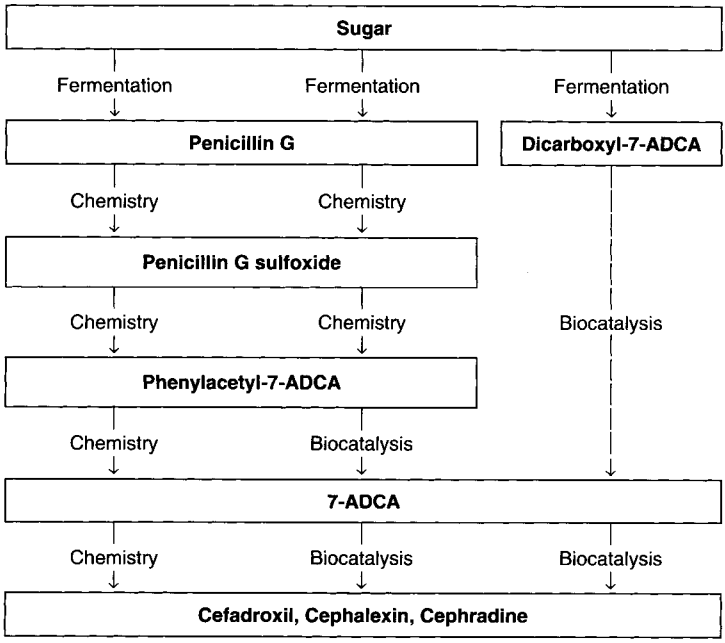


Figure 12.2-8. Production of 7-ADCA has undergone remarkable changes. In the early days (left-hand side), chemical ring expansion of penicillin G resulted in the formation of the cephalosporin nucleus. The phenylacetyl moiety was then removed chemically. Later on, this last step was replaced by a biocatalytic step using penicillin amidase (middle). On the right hand side, a completely new route is presented. Dicarboxyl-7-ADCA is obtained directly by fermentation. A dicarboxyl amidase is used to remove the dicarboxyl group.

preserve stereochemical integrity and leave the fragile four-membered β-lactam ring intact.

Today, nearly all SSAs are produced using the methodology described above. However, because of the relative complexity of these chemical processes and the use of toxic reagents and solvents, the application of biocatalysis has promising possibilities here too. Indeed, several biocatalytic processes have been developed and are now being introduced on a production scale. A noteworthy advantage of these processes is that protection of functional groups is not a prerequisite because of the mild reaction conditions and the high selectivity of the enzymes involved.

So far, the major focus of research has been directed at enzymatic coupling of D-(−)-phenylglycine methylester or D-(−)-phenylglycine amide with either 6-APA or 7-ADCA yielding ampicillin and cephalexin, respectively, and at the coupling of D-(−)-4-hydroxyphenylglycine derivatives with either 6-APA or 7-ADCA leading to amoxicillin and cefadroxil, respectively (Fig. 12.2-9)[63, 70–75]. During this kinetically controlled condensation, the activated 4-hydroxyphenylglycine forms an acyl-enzyme complex with the penicillin amidase[76]. Subsequently, this acyl-enzyme complex is deacylated by a nucleophile, the β-lactam nucleus 6-APA or 7-ADCA, or

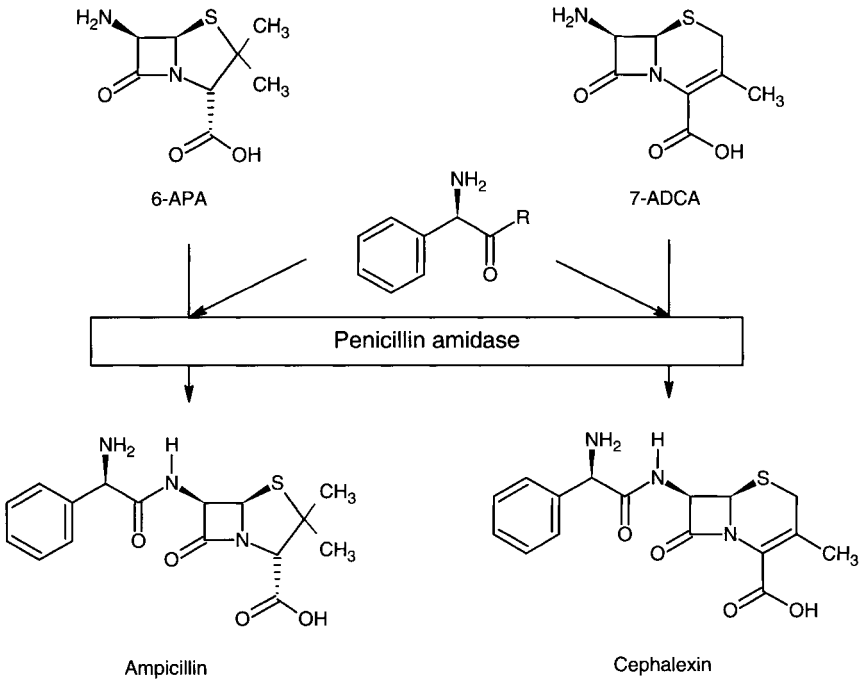


Figure 12.2-9. Enzymatic conversion of 6-APA and 7-ADCA into ampicillin and cephalexin using penicillin amidase. The side chain is introduced using an activated form of D-(-)-phenylglycine, either the amide ($R = NH_3$) or the ester ($R = OCH_3$, OC_2H_5).

water. In the first case this leads to product formation, whilst in the second case the activated side chain is hydrolyzed. By carefully tuning reaction conditions and downstream processing sequences for every individual product, yields of up to 90% based on 6-APA or 7-ADCA have been obtained. However, as a result of the competing hydrolysis of the acyl-enzyme complex by water the yield with respect to the ester or amide was quite low (approximately 30%)[72, 73]. By performing this condensation in the presence of an alcohol, as a result of which the activated phenylglycine is 'recycled' *in situ*, the yield based on phenylglycine could be improved[75]. The latest breakthroughs are the development of immobilized biocatalysts with improved performance, application of low temperatures and using high substrate concentrations[71]. It is these improvements that make enzyme-catalyzed synthesis of SSAs in a purely aqueous environment competitive industrially with traditional chemical synthesis.

12.2.6
Conclusions and Future Prospects

As indicated in the preceding sections, amides and their derivatives are important versatile building blocks for the (agro)chemical and pharmaceutical industry. Owing to the selectivity of amidases (both regio- and enantioselectivity) and the fact that

Table 12.2-4. Reduction of waste volumes resulting from introducing penicillin acylase biocatalysis in antibiotics production.

Process	Waste reduction factor
Penicillin G → 6-APA	5
Penicillin G → 7-ADCA	2
6-APA → semi-synthetic penicillins	3
7-ADCA → semi-synthetic cephalosporins	3

these conversions can be achieved under very mild conditions, several biocatalytic processes based on amidases have recently been commercialized. The use of these biocatalysts in the chemical industry is expected to increase in importance in the near future as environmental restrictions become more pronounced. The benefits in terms of reduction of waste can be enormous, as can be judged from that already achieved in the production of antibiotics (Table 12.2-4).

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12.3 Hydrolysis of *N*-Acylamino Acids

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12.3.1 Introduction

The enzymatic hydrolysis of *N*-acylamino acids has been known for a century and was first detected in aqueous kidney preparations [1]. Based on the finding that this enzymatic hydrolysis proceeds enantiospecifically [2], Greenstein and coworkers developed a general and very attractive procedure for the resolution of a vast number of racemic *N*-acylated amino acids to the corresponding L-amino acids catalyzed by aminoacylase (E.C. 3.5.1.14) whereas the *N*-acetyl-D-amino acid does not react [3] (Fig. 12.3-1).

These initial investigations on a laboratory scale subsequently lead to industrial processes for the production of L-amino acids on a multi-ton scale applied by Tanabe [4–8] and Degussa [9–12]. The first work on the isolation and characterization of aminoacylases came from Greenstein and coworkers. Fractionation of hog kidney homogenates with ammonium sulfate and acetone revealed that two distinct enzymes were present in the crude preparation [13]. One was found to hydrolyze a large number of *N*-acetylamino acids and was designated acylase I (E.C. 3.5.1.14) whereas the other was found to hydrolyze preferentially *N*-acylated L-aspartic acid and was designated acylase II (aspartoacylase, E.C. 3.5.1.15). Additionally, a third aminoacylase, which acts preferentially on *N*-acylated aromatic amino acids, was found in kidney homogenates and was designated acylase III [14, 15]. Besides the L-

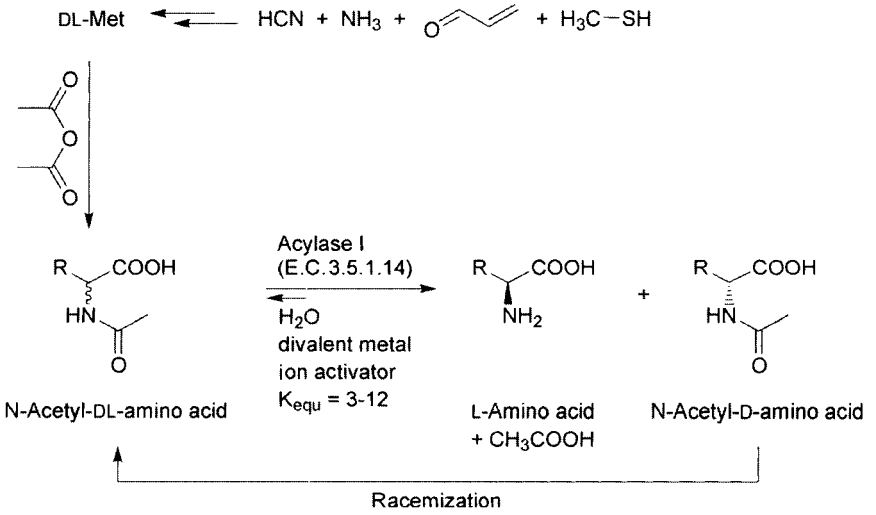


Figure 12.3-1. Enantiospecific hydrolysis of *N*-acetyl-D,L-amino acids catalyzed by aminoacylase I.

specific enzymes from kidney preparations, L- as well as D-specific aminoacylases have been isolated from a variety of microorganisms[16–33].

Although the physiological function of these enzymes is not known with certainty, it is assumed that they may be involved in the degradation of *N*-acylated amino acids occupying the *N*-termini of many proteins and are subsequently formed in the catabolic metabolism of proteins[17, 34, 35].

12.3.2
Acylase I (*N*-Acylamino Acid Amidohydrolase, E.C. 3.5.1.14)

Since acylase I has a wide substrate specificity and high enantioselectivity, it is a broadly applicable enzymatic catalyst for the kinetic resolution of most of the natural

Table 12.3-1. Substrate specificity of acylase I (E.C. 3.5.1.14) from hog kidney[13].

Substrate	Relative activity	
	X = acetyl	X = chloroacetyl
N-X-D,L-methionine	100[a]	413[b]
N-X-D,L-ethionine	64	–
N-X-D,L-norvaline	–	167
N-X-D,L-aminobutyric acid	–	139
N-X-D,L-norleucine	59	126
N-X-D,L-aminoheptanoic acid	–	117
N-X-D,L-leucine	22	68
N-X-D,L-alanine	13	48
N-X-D,L-serine	–	48
N-X-D,L-glutamic acid	13	52
N-X-D,L-aminocaprylic acid	–	32
N-X-D,L-valine	7	21
N-X-D,L-aminocyclohexylacetic acid	–	19
N-X-D,L-aminophenylacetic acid	–	19
N-X-glycine	–	11
N-X-D,L-allothreonine	–	11
N-X-D,L-threonine	–	3
N-X-D,L-isoleucine	2	–
N-X-D,L-arginine	2	–
N-X-D,L-alloisoleucine	1.0	–
N-X-D,L-histidine	0.6	–
N-X-D,L-phenylalanine	0.6	–
N-X-D,L-diaminopropionic acid	–	1.9[c]
N-X-D,L-aminocyclohexylpropionic acid	–	1.4
N-X-D,L-tyrosine	–	1.4
N-X-D,L-ornithine	–	1.3[c]
N-X-D,L-lysine	–	0.6[c]
N-X-D,L-aminocyclohexylbutyric acid	–	0.5
N-X-D,L-*S*-benzylcysteine	0.4	–
N-X-D,L-tryptophan	< 0.1	< 0.1
N-X-D,L-aspartic acid	< 0.1	< 0.1
N-X-D,L-proline	< 0.01	< 0.1
N-X-D,L-phenylserine	–	0

a 400 μmoles × min^{-1} per mg of N at 38 °C. **b** Dichloroacetyl. **c** Not determined.

Table 12.3-2. Comparison of the kinetic and chemical properties of pig kidney and *Aspergillus oryzae* aminoacylase[35].

Property	*Aspergillus oryzae* aminoacylase	Pig kidney aminoacylase
Molecular mass	73 200	85 500
Subunits	2	2
Metal ions (Zn^{2+})	6	2
SH-groups	0	4
Inhibition by *N*-α-p-tosyl-L-lysine Chloromethyl ketone HCl	–	+
Inhibition by diethylpyrocarbonate	+	+
Inactivation by metal chelating agents	completely reversible	completely reversible
pH-optimum (chloroacetylalanine)	8.5	8.0
1/2 Cystine residues	4	12
Tryptophan residues	6	12
$K_M \times 10^3$ mol L^{-1} (chloroacetylalanine)	6.3	6.6
Spec. activity U mg^{-1} (chloroacetylalanine)	319 (pH 8.0)	250 (pH 7.8)
Peptidase activity	–	+
Cl-Ac-Glu-PABA hydrolysis	+	–
Heat stability	60 °C: denaturation	60 °C: denaturation
Activation by Co^{2+}	+	+

as well as unnatural and rarely occurring α-amino acids[3, 36]. Thus it is the most important and mostly frequently used aminoacylase in the chemoenzymatic synthesis of L-amino acids from the corresponding racemic *N*-acetylated precursors (Table 12.3-1).

The numerous investigations on acylase I have been reviewed on several occasions[36]. Acylase I has been isolated and characterized from kidney preparations[13, 37–39] as well as the fungi *Aspergillus oryzae*[16, 17, 35, 40] (AA) and *Aspergillus melleus* (AM). The enzymes from the two *Aspergillus* species are virtually identical[41]. A comparison of some kinetic and chemical properties of pig kidney acylase (PKA) and the mold enzyme from *Aspergillus oryzae* (AA) is shown in Table 12.3-2. Both enzymes are dimeric, zinc-containing metalloproteins of roughly the same size and which are strongly activated by external addition of cobalt ions[17, 35, 40, 42–44]; Zn^{2+} is essential for activity[40]. The Co^{2+}/acylase-dissociation constants of PKA and AA are similar with $10^{-7.5}$ M[40] und 10^{-7} M[43], respectively, the respective constants for Zn^{2+}/acylase are identical at 10^{-10} M[40, 45]. They differ in the amount of zinc ions bound per subunit and in the number of SH-groups as well as cysteine and tryptophan residues essential for catalytic activity. The properties of acylase I from *Aspergillus oryzae* are summarized in Table 12.3-3[46].

12.3.2.1
Genes, Sequences, Structures

The DNA and protein sequences of eight aminoacylases are now known, as of March 2001. Sequences from *Homo sapiens*[47, 48], pig[49, 50], *Bacillus stearothermophilus*[51] and *Lactococcus lactis*[52] are known for aminoacylase I, sequences from *Homo*

Table 12.3-3. Properties of acylase I from *Aspergillus oryzae*[46].

Parameter	Quantity	Reference
Molar mass Da; no. of subunits	73 200; 2 (identical)	[35]
Specific activity (pure enzyme, U mg^{-1})	319 (ClAc–Ala, pH 8.0)	[35]
T; pH optimum	55 °C; pH 7.5	[15]
Substrate(s) [K_M-value(s), mM]	ClAc-Met [1.5], ClAc-Phe (0.7)	[35]
Activators (0.5 mM, in %), pH 6	Co^{2+} (151), Zn^{2+} (100), Mg^{2+} (97), Mn^{2+} (37), Ni^{2+} (27)	[35]
Inhibitors	Cd^{2+}, Cu^{2+}, chelators	[35]
Sequences and structure:		
protein sequence: not known	DNA sequence: not known	
expression system: not known	3D crystal structure: not known	

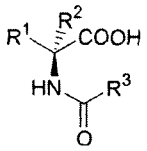
sapiens[53, 54] and *Mus musculus* (house mouse)[55] for aminoacylase II and sequences from *Achromobacter xylosoxidans* A-6[56] and *Alcaligenes faecalis*[57] for D-aminoacylase. The DNA sequences of several other acylases, notably L-acylases I, from organisms such as *Arabidopsis thaliana, Streptomyces coelicolor, Bacillus subtilis* and from two human genome project groups have been annotated as aminoacylases but have not been confirmed to possess aminoacylase activity. Regarding three-dimensional structures, as of the beginning of March 2001, no structures of aminoacylases were known or under review according to the Protein Data Bank (PDB).

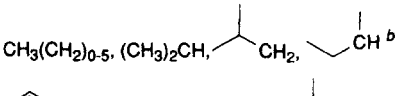
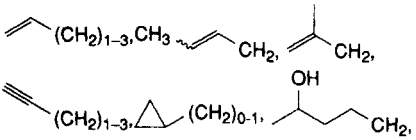
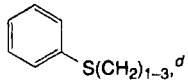
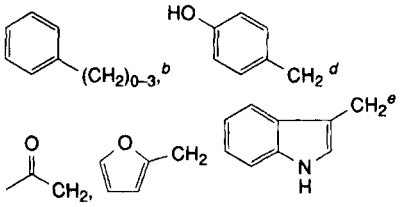
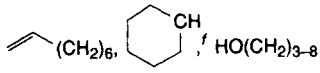
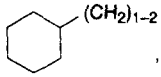
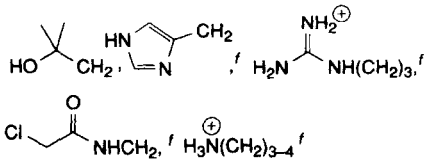
12.3.2.2
Substrate Specificity

An extensive study of the substrate specificity of both enzymes (AA, PKA), especially for the resolution of unnatural and rarely occurring amino acids has been conducted by Whitesides and coworkers[36] (Fig. 12.3-2).

Both enzymes have an unusually wide substrate specificity with a preference for hydrophobic substrates. *N*-acylated aliphatic straight-chain amino acids are the preferred substrates for both enzymes, however, the corresponding aliphatic branched-chain amino acids are also readily accepted, especially by the fungal enzyme[13, 35]. *N*-acylated amino acids with an aromatic side chain are significantly hydrolyzed only by the fungal enzyme[17] (Table 12.3-4). The substrate spectrum of AA was even broader than anticipated[58]. Sulfur- and selenium analogs react at comparable rates, often even faster than the carbon analogs; four to five atoms are the optimum length of the side chain. *S*-Methyl-L-cysteine gained significance recently as building block for HIV-protease inhibitors[59, 60], L-selenomethionine was described as part of a suitable treatment for Alzheimer's disease and Parkinson's syndrome[61]. Another striking difference is that the renal enzyme hydrolyzes dipeptides whereas the mold enzyme does not[62].

Acylase I has not only been used for the enantioselective resolution of *N*-acetyl-D,L-amino acids to the corresponding L-amino acids but also for substrate-selective resolution of *N*-acetyl amino acids using the different activity of the enzymatic



R^1	R^2	R^3	reactivity[a]
$CH_3(CH_2)_{0-5}$, $(CH_3)_2CH$, 			
			
$HOCH_2$, $ClCH_2$, $NC(CH_2)_{3-4}$, $HOOC(CH_2)_{2-3}$,[c]	H	CH_3, C_2H_5 XCH_2 (X = Cl, Br, CH_3O) XCH_2CH_2 (X = Cl, Br) H,[b] C_6H_5,[d] H_2NCH_2[d]	good, > 10 %
$CH_3(CH_2)_{0-1}S$ 			
			
$CH_3(CH_2)_6$, 	H	$CH_3(CH_2)_{0-1}CH(Cl)$[g] L-$RCH(NH_2)$[g]	fair, 1-10 %
$CH_3(CH_2)_{7-8}$,[f] $(CH_3)_3C$, 	H		
	CH_3[g]	$CH_3CH_2CH(CH_3)$[g]	poor, 0.01-1 %

a Reactivities of substituents (R^1, R^2, R^3) are expressed relative to the reactivities of the corresponding substituents of the model substrate, *N*-acetylmethionine (40 mM racemic; $R^1 = CH_3SCH_2CH_2$, R^2, R^2 = H, R^3 = CH_3. **b** AA only; reactivity with PKA is fair. **c** PKA only; reactivity with AA is fair. **d** AA only; reactivity with PKA is poor. **e** AA only; no reactivity with PKA. **f** Data for PKA only. **g** PKA only; no reactivity with AA.

Figure 12.3-2. Reactivities of substituents of acylase I[36].

Table 12.3-4. Comparison of the relative activity of *Aspergillus* and pig kidney aminoacylase with different substrates[35].

Substrate	Conc. [mM]	Aspergillus aminoacylase	Pig kidney aminoacylase
N-chloroacetyl-L-alanine	7.1	100[a]	100[a]
N-chloroacetyl-L-methionine	7.1	400	480
N-chloroacetyl-D,L-norleucine	2.1	207	120
N-chloroacetyl-L-leucine	2.1	26	96
N-chloroacetyl-L-phenylalanine	3.5	325	5
N-chloroacetyl-L-tryptophan	2.1	125	0
N-acetyl-L-glutamic acid	8.2	0	21
N-acetyl-L-aspartic acid	8.2	0	0
N-acetyl-L-glutamine	8.2	13	4
N-acetyl-L-alanine	8.2	14	7
N-acetyl-L-lysine	8.2	3	0
N-dichloroacetyl-glycine	4.1	0	1
N-dichloroacetyl-L-leucine	4.1	0	3
N-dichloroacetyl-D,L-norleucine	4.1	4	69
N-dichloroacetyl-L-alanine	4.1	0.7	2

a $V_{max} = 4.2\ \mu M\ s^{-1}$.

catalyst towards different *N*-acetyl-L-amino acids. Martens and Weigel used kidney acylase for the separation of *N*-acetyl-L-leucine and *N*-acetyl-L-isoleucine[63].

12.3.2.3
Stability of Acylases

Acylase I from both sources is very stable as a lyophilized powder. In aqueous solution, the resting stability of acylase from *Aspergillus oryzae* was found to depend much more on pH than on concentration: while at room temperature (25 °C) and standard pH (7.0) the half-life $\tau_{1/2}$ was around 60 d for concentrations of between 30 and 120 g crude enzyme L^{-1}, the $\tau_{1/2}$ dropped to 45 d at pH 6.5 and to about 30 d at pH 6.0[46]. Also, in solubilized form, the fungal enzyme is fairly stable whereas the pig kidney enzyme is sensitive to auto oxidation and therefore should be kept under nitrogen if stored in a solubilized form[36]. Tests for operating stability in repeated-batch mode[9, 17, 36, 64] reveal that acylase from *Aspergillus oryzae* again fares much better than the porcine kidney enzyme. Tests for operating stability in a continuous reactor with the acylase from *Aspergillus oryzae*[65] again demonstrated:

- superior stability of AA (616 U kg^{-1} L-methionine) over PKA (6000 U kg^{-1} L-met), both measured with $[Co^{2+}]$ at 5×10^{-4} M,
- tighter binding of Zn^{2+} vs. Co^{2+} at 5×10^{-4} M (308 vs. 616 U kg^{-1} L-met),
- that loss of metal, commonly Zn^{2+}, is responsible for activity loss and
- possibility of reconstitution over a timescale of several hours, whereas the time constant of leaching is on the order of 48 h, as well as
- the option of pulsing divalent metal addition resulting in 477 U kg^{-1} L-met at $[Zn^{2+}]$ of 4×10^{-4} M.

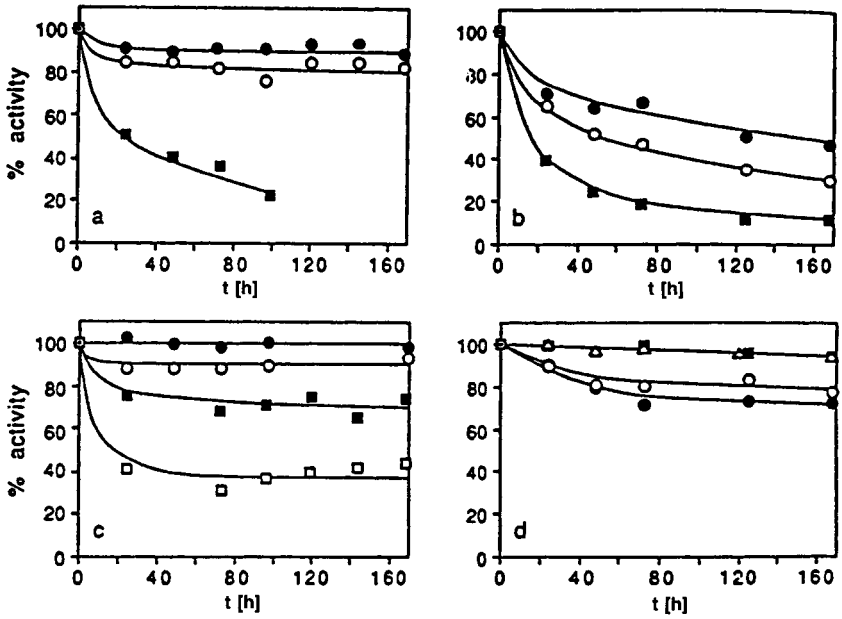


Figure 12.3-3. Stability of soluble acylase I in the presence of organic cosolvents. Acylase I from porcine kidney (a, c) and from *Aspergillus* (b, d) were incubated in 0,10 M phosphate buffer, pH 7.5, containing ethanol (a, b) or DMSO (c, d), at 25 °C, under nitrogen. Concentrations of organic cosolvents were 10% (●), 20% (○), 30% (■), 40% (□), and 50% (△) [160].

Porcine kidney acylase seems to have a different spectrum of activation [64]: although Co^{2+} activates PKA most strongly, Ca^{2+} is not far behind whereas Zn^{2+}, just like Mg^{2+} or Fe^{2+}, does not seem to exert a strong effect. Both enzymes have moderate thermostability [35] and moderate stability in the presence of organic cosolvents [36] (Fig. 12.3-3).

The behavior of aminoacylase both from porcine kidney and *Aspergillus* sp. towards a wide range of water-miscible cosolvents was investigated by Iborra et al. [66]. They found that enzymatic activity can be correlated with the denaturing capacity of the water-cosolvent system.

In 1993, a thermostable aminoacylase from *Bacillus stearothermophilus* was characterized by Sakanyan et al. [51]. The enzyme hydrolyzes *N*-acyl derivatives of aromatic amino acids preferentially and even has some dipeptidase activity. Its optimal reaction temperature is 70 °C; after incubation for 15 min, 90% of the original activity was retained. The authors write that the similarity of the *B. stearothermophilus* enzyme sequence with that of other enzymes such as aminoacylase I, acetylornithine deacetylase and carboxypeptidase G2 suggests a common origin. The aminoacylase from *B. stearothermophilus* is well characterized: the gene has been completely sequenced [51], cloned into *E. coli* and overexpressed [51, 67] and studied for catalytic and stability properties [67]: the intrinsic one Zn^{2+} ion per subunit seems to have a predominantly structural role and activity can be restored to the apo-enzyme by Co^{2+} and particularly by Cd^{2+} (3-fold activity!) but not by Zn^{2+}.

Table 12.3-5. Thermodynamics of the *N*-acetyl amino acid reaction[69]. Conditions: pH 7.5, T = 25 °C; x_{eq} calculated at $[S_0]$ of 0.5 M, K_{eq}-data determined from synthesis and hydrolysis reaction.

Amino acid	K_{eq}	x_{eq}
Glycine	4.5	90.8%
Alanine	5.6	92.4%
Aminobutyric acid	5.6	92.4%
Norvaline	10.5	95.6%
Norleucine	12.5	96.3%
Methionine	3.7	89.2%

Another thermostable acylase, aminoacylase SK-1, was reported by the Amano Pharmaceutical Comp.[68]. The enzyme is isolated from *B. stearothermophilus* IFO 12983. It possesses an optimal temperature for reaction at 60 °C and is stable at 70 °C for at least 30 min. The preferred substrates are dipeptides besides the *N*-acyl derivatives of Met, Phe and Tyr. K. Soda's group has isolated and characterized a thermostable aminoacylase from *Bacillus thermoglucosidius*[27] which has many similarities to the *Aspergillus* enzyme, such as metal content and requirements, activity and specificity profile as well as high stability at elevated temperatures and high content of organic solvents and denaturants. Judged by the identity of the organism used for culturing, of the specificity profile and of some enzyme properties (both are identical dimers with molecular mass of 86000 Da), aminoacylase SK-1 and the aminoacylase from *Bacillus thermoglucosidius*[27] seem to be the same enzyme.

12.3.2.4
Thermodynamics and Mechanism of the Acylase-catalyzed Reaction

Equilibrium

The hydrolysis reaction of *N*-acetyl amino acids is equilibrium-limited, however, the equilibrium is well on the side of the hydrolysis so that at low substrate concentrations conversion is almost quantitative. For the case of N-acetyl methionine, Wandrey and Flaschel determined the equilibrium constant K defined as in Eq. (1)

$$K = \frac{[\text{acetate}][\text{L-Met}]}{[N\text{-Ac-L-Met}]} \tag{1}$$

and found K = 2.75 M at 37 °C and pH 7[9]. Then, at 37 °C and $[S_0]$ = 100 mM, equilibrium conversion x_e is 96% (based on *N*-Ac-L-Met), at $[S_0]$ = 500 mM, x_e = 86%. The enthalpy of reaction is 7.9 kJ mol^{-1}[9]. Data for other substrates are listed in Table 12.3-5. More physicochemical data on the *N*-acetyl amino acid acylase reaction can be found in ref.[64].

pH-Dependence

The Michaelis constant for hydrolysis is independent of pH in the pH range 6.0–9.5 whereas the pH-dependence of maximum velocity has a bell-shaped profile with the maximum at pH 7.5 and inflection points at p*K*a values of 6.7 and 8.9[69].

Table 12.3-6. Substrate specificity of acylase II (aspartoacylase; E.C. 3.5.1.15) from hog kidney[13].

Substrate	Relative activity	
	X = acetyl	X = chloroacetyl
N-X-D,L-aspartic acid	100[a]	526
N-X-D,L-glutamic acid	–[b]	22
N-X-D,L-methionine	33	–
N-X-D,L-alanine	–	19
N-X-D,L-leucine	–	26
N-X-D,L-serine	–	11

a 0.45 μmoles×min^{-1} per mg of N at 38 °C. **b** Not determined.

Mechanism

The mechanism of acylase-catalyzed reaction has been studied, particularly for porcine kidney acylase[34, 69–71]. While the mechanism of action was contested for some time between a linear mechanism (random uni-bi)[34, 69, 70] and a double-displacement, "ping-pong", mechanism involving a stable intermediate[71], it now seems to have been decided that base-catalyzed attack of the carbonyl carbon by water is the rate-determining step followed by a linear sequence involving an E-P_1P_2-complex[34, 69, 70], Eq. (2):

$$E + A \overset{H_2O}{\rightleftharpoons} E\text{-}P_1P_2 \overset{P_1}{\rightleftharpoons} E\text{-}P_2 \overset{P_2}{\rightleftharpoons} E \qquad (2)$$

Recent work on the non-competitive inhibition of both porcine and fungal aminoacylase by α- and β-fluoro- and -hydroxy acids indicated that the active site of the fungal enzyme should interact with the α-substituent of a substrate via an acidic group while the porcine enzyme has a basic group in the corresponding position with which to recognize substrates[72].

Enantiospecificity

Acylase I acts on racemates in a highly enantiospecific way to yield L-amino acids exclusively, with ee values in almost all cases, especially with *N*-acetyl substrates, exceeding 95%. According to Cahn-Ingold-Prelog rules, L-amino acids correspond to the (S)-configuration, with the exception of L-cysteine which is in the (*R*)-configuration owing to first stereochemical priority of the thiomethyl group. In general, the amino acid amide enantiomer bearing the larger substituent in the pro-(*S*)-position is hydrolyzed preferentially[73].

12.3.3
Acylase II (*N*-Acyl-L-Aspartate Amidohydrolase, Aspartoacylase, E.C. 3.5.1.15)

Apart from acylase I, another aminoacylase was found in kidney preparations by fractionation of hog kidney homogenates with ammonium sulfate and acetone[13]. Whereas acylase I could be enriched and thus partially purified by this procedure the main activity with *N*-acylated-L-aspartic acid as the substrate was found in another

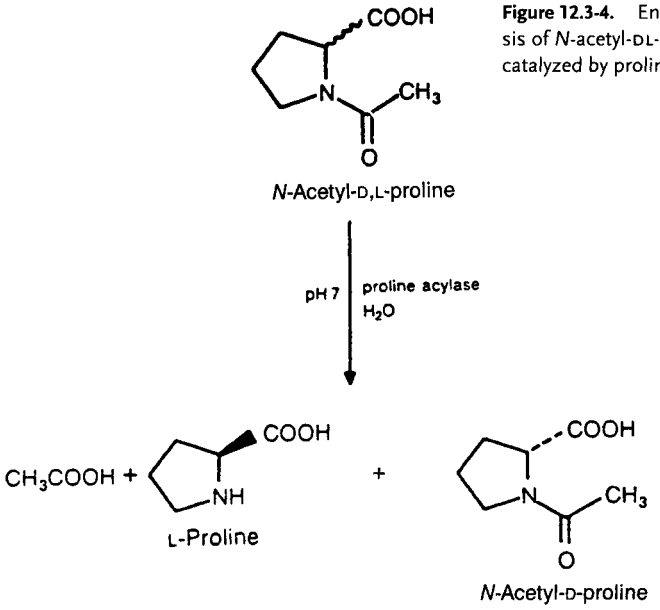


Figure 12.3-4. Enantioselective hydrolysis of *N*-acetyl-DL-proline to L-proline catalyzed by proline acylase[76].

Table 12.3-7. Comparison of some kinetic and chemical properties of proline acylases from three different microorganisms[19–22].

Property	*Ps. spec.*[19]	*Rh. rubra*[20]	*Com. Testosteroni*[21, 22]
Enantiospecificity	L	L	L
Molecular mass Da	597 000 +/– 12000	560 000	380 000 +/– 40 000
No. of subunits	10–12	–[a]	8
Molecular mass of subunits (Da)	55 000	–	45 000 +/– 15 000
Isoelectric point	5.0	–	–
pH-optimum	6.0	6.0	6.8
pH-stability	7.0–8.0 (30 min, 35 °C)	6.0–10.0	5.0–10.0 (4 weeks, room temp.)
Temp. optimum	–	50 °C	65 °C
Temp. stability	40 °C (10 min, pH 8.0)	40 °C	70 °C (30 min, pH 7.5)
Activation by divalent cations	none	none	none
Inhibitors	phosphate EDTA *o*-phenanthroline 2,2-dipyridyl $Hg^{2+} > Cu^{2+} > Zn^{2+} > Fe^{3+} > Ni^{2+} > Pb^{2+}$	PCMB Cu^{2+}	phosphate 2-mercaptoethanol *o*-phenanthroline PCMB, PHMB $Fe^{2+} > Hg^{2+} > Cu^{2+} > Zn^{2+} > Sn^{2+} > Fe^{3+}$
Reactivation of the apoenzyme by divalent cations	$Mn^{2+} > Ca^{2+}$ $Pb^{2+} > Co^{2+}$ $Zn^{2+} > Ba^{2+}$	–	$Co^{2+} > Zn^{2+}$

a Not determined.

fraction of the ammonium sulfate precipitates. To distinguish between these two activities, the former fraction was designated acylase I, and the latter acylase II [13]. In contrast to acylase I, acylase II has a very narrow substrate specificity. Among the *N*-acetyl derivatives of the twenty proteinogenic amino acids only *N*-acetyl-L-aspartic acid is hydrolyzed significantly (Table 12.3-6). Therefore, acylase II from kidney preparations was designated as aspartoacylase or *N*-acyl-L-aspartate amidohydrolase (E.C. 3.5.1.15) and is the enzyme of choice for the resolution of racemic aspartic acid.

Table 12.3-8. Comparison of the substrate specificity of proline acylases from four different microorganisms [18–21].

	Relative activity			
Substrate	***Alc. spec.*** [18]	***Ps. spec.*** [19]	***Rh. rubra*** [20]	***Com. testost.*** [21]
N-acetyl-L-Pro	100[a]	100[b]	100	100[c]
N-acetyl-D-Pro	0	0	0	0
N-acetyl-L-Ala	–[d]	0.03	–	9
N-acetyl-D,L-Ser	–	0	–	0.2
N-acetyl-L-Val	–	–	–	0.2
N-acetyl-D,L-Val	–	0	–	–
N-chloroacetyl-L-Pro	–	172	–	362
N-chloroacetyl-L-Met	–	–	–	17
N-chloroacetyl-L-Val	–	–	–	14
N-chloroacetyl-L-Leu	–	–	–	2
N-chloroacetyl-L-Phe	–	–	–	1
N-chloroacetyl-L-Tyr	–	–	–	1
N-chloroacetyl-L-Ile	–	–	–	0.5
N-acetyl-L-Hyp	–	–	–	10
N-formyl-L-Pro	–	–	–	18
N-propionyl-L-Pro	–	13	–	29
N-butyryl-L-Pro	40	24	–	14
N-valeryl-L-Pro	–	–	–	15
N-caproyl-L-Pro	12	33	–	9
N-capryloyl-L-Pro	–	23	–	–
N-caprinoyl-L-Pro	–	59	–	–
N-myristoyl-L-Pro	–	7	–	–
N-palmitoyl-L-Pro	13	0.6	–	–
N-benzoyl-L-Pro	–	61	–	–
Gly-L-Pro	67	123	0	4
N-Z-L-Pro[e]	32	24	4	0
N-Z-Gly-L-Pro	146	269	208	11
N-Z-L-Ala-L-Pro	0.6	2	–	–
N-Z-Gly-L-Ala	–	1	–	–
N-Z-Gly-L-Pro	–	217	–	–
N-Z-Gly-L-Pro				
L-Leu-Gly-L-Pro	102	101	0	–

a 142 μmoles × min^{-1} × mg^{-1}. **b** 410 μmoles × min^{-1} × mg^{-1}. **c** 85 μmoles × min^{-1} × mg^{-1}.
d Not determined. **e** Z: benzyloxycarbonyl.

12.3.4
Proline Acylase (*N*-Acyl-L-Proline Amidohydrolase)

The acylase-catalyzed resolution of *N*-acyl-D,L-amino acids has some limitations. Although acylase I from porcine kidney and *Aspergillus oryzae* has a broad substrate specificity and high enantioselectivity, the enzyme does not accept *N*-acylated substrates where the hydrogen atom at the amide nitrogen is replaced by an alkyl group. Therefore, *N*-acylated secondary amines such as *N*-acetyl-proline and *N*-acetyl-*N*-alkyl-amino acids are not hydrolyzed by this enzyme[13, 36, 37, 74, 75] as well as aminoacylases from other sources[32]. This gap in the substrate specificity of aminoacylase I was successfully closed with the isolation of acylases which act specifically on *N*-acetyl-L-proline and its derivatives[18–22] (Fig. 12.3-4).

The enzyme has been isolated from *Alcaligenes* sp.[18, 21], *Pseudomonas* sp.[19], *Rhodotorula rubra*[20] and *Comamonas testosteroni*[21, 22]. Some kinetic and chemical properties of proline acylases from three different microorganisms are listed in Table 12.3-7. A comparison of the substrate specificity of proline acylases from four different microorganisms is shown in Table 12.3-8.

Proline acylase is a relatively large protein with a molecular mass in the range of 380–600 kDa consisting of 8–12 subunits with a molecular mass of 45–55 kDa. The

Table 12.3-9. Substrate specificity of proline acylases from *Comamonas testosteroni* towards *N*-acyl-amino acids[77].

Substrate	Relative activity	Conversion (%)
N-acetyl-L-proline	100	100
N-acetyl-D,L-proline	78	54
N-acetyl-L-thiazolidine-4-carboxylic acid	255	99
N-acetyl-L-azetidine-2-carboxylic acid	< 50	100
N-acetyl-D,L-pipecolic acid	58	49
N-chloroacetyl-L-proline	308	100
N-chloroacetyl-D,L-proline	290	52
N-chloroacetyl-L-thiazolidine-4-carboxylic acid	357	100
N-chloroacetyl-L-azetidine-2-carboxylic acid	437	100
N-chloroacetyl-D,L-pipecolic acid	507	54
N-chloroacetyl-D,L-indoline-2-carboxylic acid	0	0

Table 12.3-10. Substrate specificity of proline acylases from *Comamonas testosteroni* towards *N*-alkyl-amino acids[78].

Substrate	Relative activity	Conversion (%)
N-chloroacetyl-L-proline	100	100
N-chloroacetyl-*N*-methyl-L-alanine	175	100
N-chloroacetyl-*N*-methyl-D,L-alanine	115	49
N-chloroacetyl-*N*-ethyl-D,L-alanine	82	–[a]
N-chloroacetyl-*N*-propyl-D,L-alanine	27	–
N-chloroacetyl-*N*-methyl-D,L-2-aminobutyric acid	10	50
N-chloroacetyl-*N*-ethyl-D,L-2-aminobutyric acid	2	–

a Not determined.

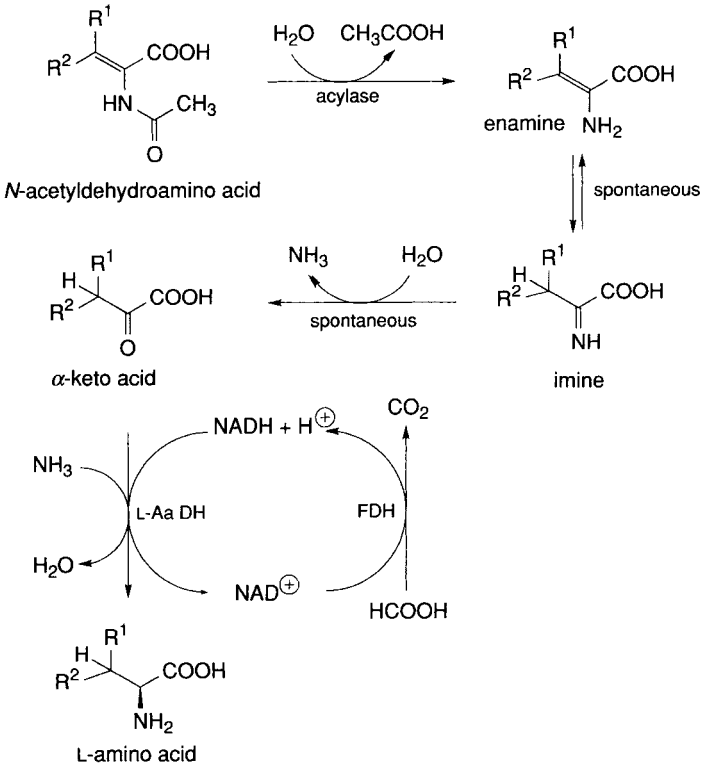


Figure 12.3-5. Coupled enzymatic reaction of a dehydro amino acid acylase with a amino acid dehydrogenase (from [82]).

enzyme is not activated by cobalt ions and has a relatively narrow substrate spectrum. The enzyme from *Comamonas testosteroni* preferentially hydrolyzes *N*-acylated L-proline and the *N*-acetyl derivatives of other cyclic imino acids [77] (Table 12.3-9) and opens for the first time the route to resolution of racemic N-acylated N-alkyl-amino acids [78] (Table 12.3-10). Among the proteinogenic amino acids, only the *N*-acetyl derivatives of L-proline and L-alanine are hydrolyzed to a significant extent [21, 22, 77].

12.3.5
Dehydroamino Acid Acylases

A new acylase was found in strains of *Brevibacterium* sp. by Hummel [79, 80] in 1987, catalyzing the hydrolysis of acetamidocinnamate (ACA) and was named acetamidocinnamate acylase (ACA acylase). A similar, just as enantiounspecific acylase, *N*-acetyldehydroleucine acylase (ACL acylase), catalyzing *N*-acyl hydrolysis of branched-chain dehydroamino acids (*N*-acetyl-dehydrovaline, -leucine and -isoleucine) was isolated and characterized from *Zoogloea ramigera* by Kittelmann and Kula [81, 82]. The hydrolysis product in both cases, an enamine, first undergoes

Table 12.3-11. Comparison of the substrate specificity of D-, and L-aminoacylase in *Streptomyces tuirus* and *Streptomyces olivaceus*[28].

	Relative activity			
	S. tuirus		*S. olivaceus*	
Substrate	D-	L-	D-	L-
N-acetyl-phenylglycine	100	–[a]	100	–
N-acetyl-leucine	1130	70	957	120
N-acetyl-phenylalanine	1004	8	723	15
N-acetyl-methionine	682	167	448	208
N-acetyl-tyrosine	522	0	307	2
N-acetyl-valine	314	29	261	61
N-acetyl-tryptophan	117	0	68	0
N-acetyl-alanine	102	38	69	92
N-acetyl-glutamic acid	69	0	38	10
N-acetyl-aspartic acid	20	0	0	21
N-acetyl-arginine	14	32	5	72
N-acetyl-proline	0	0	0	0

a Not determined.

spontaneous rearrangement to the ketimine which is then deaminated spontaneously to the α-keto acid. The dehydroamino acid acylase reaction can be coupled with reductive amination by amino acid dehydrogenases such as PheDH, LeuDH or even AlaDH, respectively, to produce L-amino acids[82] (Fig. 12.3-5).

L-Phenylalanine has been produced continuously from ACA with the help of ACA acylase in an enzyme membrane reactor (EMR) with a space-time-yield of 277 g L^{-1} d^{-1}[83]. With ACL acylase, L-leucine was produced at 123–180 g L^{-1} d^{-1} in the same reactor set-up[82]. The dehydroamino acid substrates can be prepared conveniently, either from 2-halogen carboxylic acid esters[84], or, specifically in the case of ACA, via the acetamidomalonic ester route by reaction with benzyl halogenides[85].

Apart from the L-specific acylases from kidney and *Aspergillus* strains it has been shown that similar aminoacylases are widely distributed in microorganisms[23–27, 86]. However, from the viewpoint of costs, those acylases which are practically employed for large scale industrial purposes, are restricted to the enzyme from *Aspergillus oryzae* (see Sect. 12.3.7).

12.3.6
D-Specific Aminoacylases

D-Specific aminoacylases have been found in *Pseudomonas* sp.[33, 87–90], *Streptomyces* sp.[28] and *Alcaligenes* sp.[29–32, 90, 91]. The first investigations on the use of D-specific aminoacylases for the synthesis of D-amino acids were carried out by Kameda and coworkers. They demonstrated that a strain of the genus *Pseudomonas* hydrolyzed *N*-benzoyl-D-amino acids in addition to *N*-benzoyl-L-amino acids[87]. The partially purified enzyme was employed to synthesize D-phenylalanine from *N*-benzoyl-D,L-phenylalanine[88] and D-phenylglycine was synthesized from *N*-chloroacetyl-D,L-phenylglycine with the crude enzyme preparation[89].

Sugie and Suzuki conducted an extensive screening among soil samples as well as

Table 12.3-12. Comparison of the substrate specificity of purified D-aminoacylases from three strains of *Alcaligenes* sp.[29, 30, 32].

	Relative activity of strain					
	DA1		DA181		MI-4	
Substrate	D-	L-	D-	L-	D-	L-
N-acetyl-methionine	100	< 1	100	0.1	100	0
N-acetyl-phenylalanine	65	< 1	81	0.3	80	0
N-acetyl-norleucine	–[a]	–	–	–	38 (D,L)	0
N-acetyl-leucine	52	< 1	60	0.4	17	0
N-acetyl-tryptophan	14	< 1	33	0.6	5	0
N-acetyl-alanine	14	< 1	25	0.8	1	0
N-acetyl-asparagine	8	< 1	16	0	0	0
N-acetyl-allo isoleucine	–	–	12	–	1	–
N-acetyl-valine	6	< 1	6	0	1	0
N-acetyl-phenylglycine	3	< 1	5	–	0	–
N-acetyl-tyrosine	–	–	–	0	–	–
N-acetyl-aspartic acid	–	–	–	0	0 (D,L)	0
N-acetyl-glutamic acid	–	–	–	0	0	0
N-acetyl-lysine	–	–	–	0	–	–
N-acetyl-arginine	–	–	–	0	–	–
N-acetyl-histidine	–	–	–	0	–	–
N-acetyl-serine	–	–	–	–	0 (D,L)	
N-acetyl-glycine	–		–		0	
N-chloroacetyl-phenylalanine	–	–	–	–	68	–
N-chloroacetyl-norleucine	–	–	–	–	66 (D,L)	–
N-chloroacetyl-isoleucine	–	–	–	–	40 (D,L)	–
N-chloroacetyl-alanine	–	–	–	–	38	–
N-chloroacetyl-valine	33	< 1	66	0	18	–
N-chloroacetyl-serine	–	–	–	–	5 (D,L)	–
N-formyl-methionine	–	–	–	–	56 (D,L)	0
N-formyl-phenylalanine	–	–	–	–	35	0
N-benzyloxycarbonyl-methionine	–	–	–	–	2	0
Glycyl-leucine	–	–	–	–	20	–

a Not determined.

among 420 strains of the genus *Streptomyces* and 16 strains of the genus *Streptoverticillium* from type culture collections and isolated four *Streptomyces* strains producing a D-specific aminoacylase suitable for the production of D-phenylglycine[28]. Since the bacteria also produced an L-aminoacylase the D-aminoacylase had to be separated from the L-specific enzyme by ion exchange chromatography prior to use. Thus, D-phenylglycine could be produced from *N*-acetyl-D,L-phenylglycine in 99.9% optical purity. Table 12.3-11 lists the substrate specificity of the D- and L-aminoacylases from two *Streptomyces* species.

Microbial D-aminoacylases have also been found in different species and strains of the genus *Alcaligenes*. The enzyme has been isolated, purified and characterized from *Alcaligenes denitrificans* subsp. *xylosoxydans*[29, 30, 32], *Alcaligenes denitrificans*[90] and *Alcaligenes faecalis*[91]. Several companies, all of them Japanese, have filed applications for D-aminoacylases recently[92–94]. The substrate specificity of the D-aminoacylases from these strains is shown in Table 12.3-12.

Table 12.3-13. Enantioselective deprotection of *N*-protected D,L-amino acids by D-aminoacylase from *Alcaligenes faecalis* DA-1 [96].

Substrate[a]	Reaction time (h)	Conversion (%)	e e of D-amino acid (%)
N-Ac-D,L-methionine	2	50.0	100
N-Ac-D,L-methionine (in 50% DMSO)	15	53.0	30
N-Ac-D,L-leucine	2	49.3	100
N-Ac-D,L-leucine (in 50% DMSO)	15	30.7/48.9	100
N-Ac-D,L-phenylalanine	2	49.9	100
N-Ac-glycine	2	10	–
N-*n*-Butyl-D,L-methionine	2	45	100
N-Bz-D,L-methionine	10	47.2	89
N-Bz-D,L-leucine	10	48.1	99
N-Bz-D,L-phenylalanine	10	50	100
N-Bz-D,L-norleucine	10	43.9	53
N-Bz-D,L-2-amino-n-butyric acid	10	33.8	80
N-Z-D,L-methionine	10	32.6	99
N-Z-D,L-leucine	10	32.6	100
N-Z-D,L-norleucine	10	12.8	51
N-Z-D,L-2-amino-*n*-butyric acid	10	15.8	77

a Ac; acetyl; Bz; benzoyl; Z; benzyloxycarbonyl.

As with the D-aminoacylases from *Streptomyces* sp. the enzymes from *Alcaligenes* strains have a preference for hydrophobic *N*-acetyl-amino acids. In this respect, they are similar to the L-specific acylase I from kidney preparations and *Aspergillus* sp. The *Alcaligenes faecalis* enzyme prefers the *N*-acyl-D-amino acid derivatives from Met, Phe and Leu [95]. If a high-affinity substrate residue occupies the hydrophobic side-chain pocket the enzyme even deacylates D-Met methyl esters or *N*-Ac-D-Met-Xaa dipeptide derivatives.

Two D-aminoacylases have been described that resemble the L-specific acylase II from kidney, which only hydrolyzes the *N*-acyl derivatives of L-aspartic acid. The D-specific counterpart of acylase II, *N*-acetyl-D-aspartate deacetylase, has been isolated from *Alcaligenes xylosoxydans* subsp. *xylosoxydans* [31]. The same strain produces an aminoacylase which specifically hydrolyzes *N*-acyl derivatives of D-glutamic acid [31]. The latter *N*-acetyl-D-glutamate deacetylase has also been found in *Pseudomonas* sp. [33].

All microorganisms producing D-aminoacylases commonly produce L-aminoacylases as well. Therefore, to reach high optical purity of the D-amino acids produced from the respective *N*-acetyl-D,L-amino acids, the D-aminoacylases have to be separated from the L-aminoacylases (Table 12.3-13). However, this is a disadvantage in view of an industrial application since additional purification steps lead to more expensive enzymes and thus add costs to the whole production process. This is one of several reasons why it is widely accepted today that the production of D-amino acids by enzyme-catalyzed hydrolysis of D,L-hydantoins seems to be more promising than the D-aminoacylase route via *N*-acetyl-D,L-amino acids. The enzyme-catalyzed synthesis of D-amino acids from the respective D,L-hydantoins is described in Chapter 12.4.

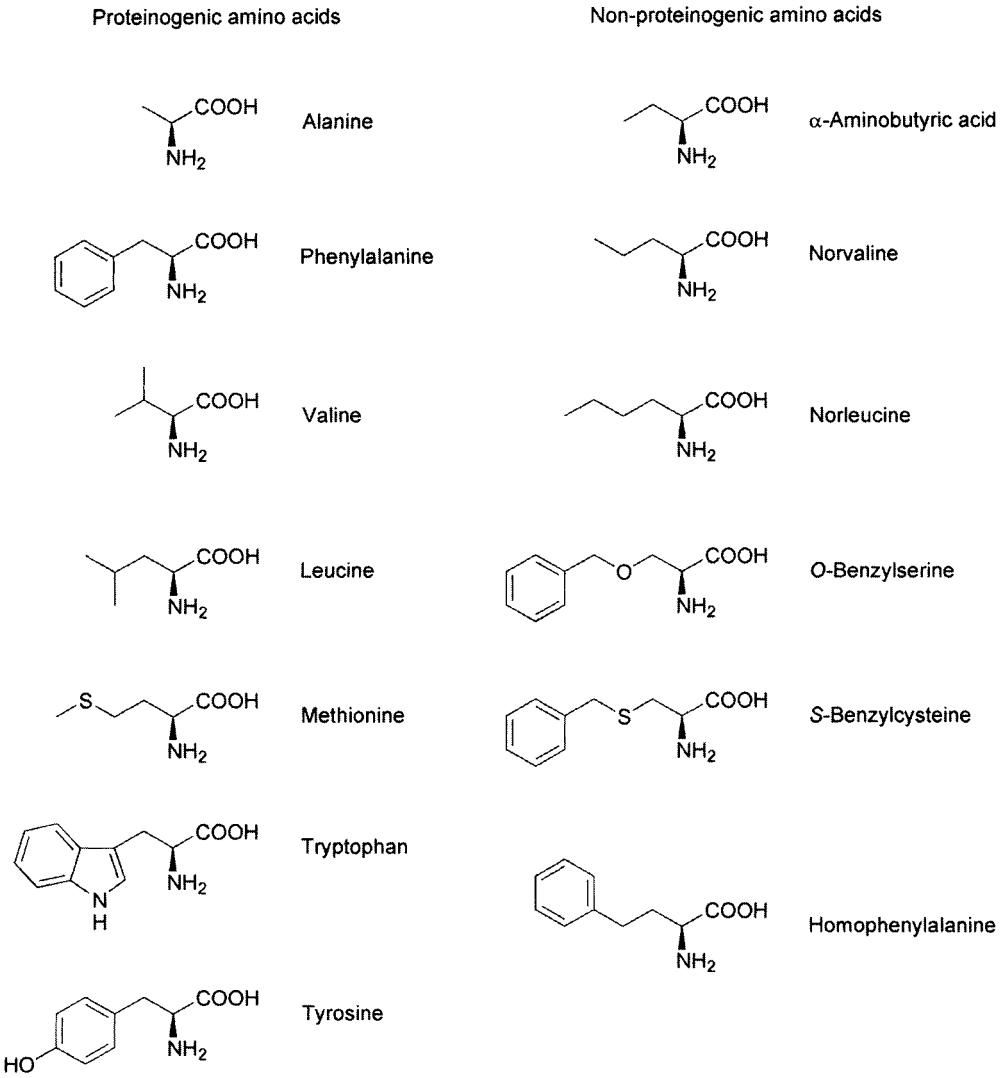


Figure 12.3-6. L-amino acids prepared in bulk quantities by acylase I resolution of *N*-acetyl-DL-amino acids.

12.3.7

Acylase Process on a Large Scale

The most established method for enzymatic L-amino acid synthesis is the resolution of racemates of *N*-acetylamino acids by acylase I from *Aspergillus oryzae* fungus. The *N*-acetyl-L-amino acid is cleaved to yield L-amino acid whereas the *N*-acetyl-D-amino acid does not react. After separation of the L-amino acid through ion exchange chromatography or crystallization, the remaining *N*-acetyl-D-amino acid can be

racemized by acetic anhydride in alkaline solution or by adding a racemase[97] to achieve very high overall conversions into the L-amino acid. *N*-acetyl-D,L-amino acids are conveniently accessible on a laboratory as well as an industrial scale through acetylation of D,L-amino acids with acetyl chloride or acetic anhydride in a Schotten-Baumann reaction[98]. As was demonstrated in the synthesis of ^{13}C-L-methionine, the acylase process has a virtually closed material balance because almost 99.5% of the amino acid components can be retrieved after processing[45].

The acylase is relevant for enzyme reaction engineering along two different lines as follows. With the aminoacylase process, Tanabe Seiyaku commercialized the first immobilized enzyme reactor system ever in 1969 after running the process in batch mode since 1954[5, 6]. Enzyme from *Aspergillus oryzae* fungus was immobilized by ionic binding to DEAE-Sephadex[4]. In a fixed-bed reactor, the reaction is carried out at elevated temperature to produce L-methionine, L-valine, and L-phenylalanine. Costs are significantly lower than in a batch process with native enzyme. Tanabe started up more fixed-bed reactor processes with immobilized enzymes: L-aspartic acid with aspartase in 1973 and L-malic acid with fumarase one year later[99–101].

At Degussa, several enzyme membrane reactor (EMR) set-ups are in operation covering six orders of magnitude from laboratory via pilot stage to full production scale; the process has been scaled up to an annual production level of several 100 tons of enantiomerically pure α-amino acids, mostly L-methionine and L-valine[102] (Fig. 12.3-6). The enzyme membrane reactor is a recycle reactor operated as a CSTR with a recycle ratio $F_{recycle}/F_{influx}$ of up to 200. For both pilot and large-scale operation, the necessary membrane area is configured into polysulfone hollow-fiber modules with a molecular-weight cut-off of 10 kDa resulting in a rejection rate of the 73 kD-acylase far in excess of 99.9%.

Aminoacylase has also been immobilized on a nylon membrane[66]. While the half-life as measured by thermal stability, of 161 d is superior to the data for immobilized acylase (65 d)[6] or soluble enzyme in an EMR[9], reactor productivity at 0.136 L-valine $kg/L^{-1}d^{-1}$ is lower than that for DEAE-Sephadex-immobilized acylase (0.5 $kg/L^{-1}d^{-1}$)[6] or that for a membrane reactor (0.35 $kg/L^{-1}d^{-1}$)[9].

Results on operational stability of both acylases in a recycle reactor at constant conversion[64] with reaction conditions close to intended large-scale conditions demonstrated much better stability of the *Aspergillus* enzyme, while renal enzyme is not stable enough for long-term operation[64,65]. Moreover, on the process scale achieved today the supply of renal acylase is insufficient, so that fungal acylase is used almost exclusively nowadays, especially since the price per unit is comparable.

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12.4
Hydrolysis and Formation of Hydantoins

Markus Pietzsch and Christoph Syldatk

12.4.1
Classification and Natural Occurrence of Hydantoin Cleaving and Related Enzymes

Abbreviations

Cit	citrulline
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
HIC	hydrophobic interaction chromatography
MTEH	methylthioethylhydantoin
IEX	ion exchange chromatography
IMH	indolylmethylhydantoin
Phg	phenylglycine
SEC	size exclusion chromatography
Thienylala	thienylalanine
O-Me-Ser	*O*-methylserine

The compound hydantoin was discovered by von Baeyer in 1861 by reduction or hydrogenation of allantoin, which is a naturally occurring cyclic amide in many plants[1]. The systematic terms for "hydantoin" are "imidazolidine-2,4-dione" or "2,4-diketotetrahydroimidazole". In the literature, a wide spectrum of various 5-mono- and 5,5'-disubstituted hydantoin derivatives of industrial and pharmacological interest is described, of which 5-monosubstituted hydantoins may be regarded as cyclic ureides of α-amino acids. They are obtained by Strecker synthesis and are important precursors, e.g. in the industrial production of D,L-α-amino acids. The 5,5'-disubstituted hydantoin derivatives have been of pharmacological interest since the 1930s, e.g. for the treatment of Parkinson's disease. Figure 12.4-1 gives a survey of the different methods for the chemical synthesis of hydantoins. Detailed reviews on their chemical syntheses and applications are given in references[2]

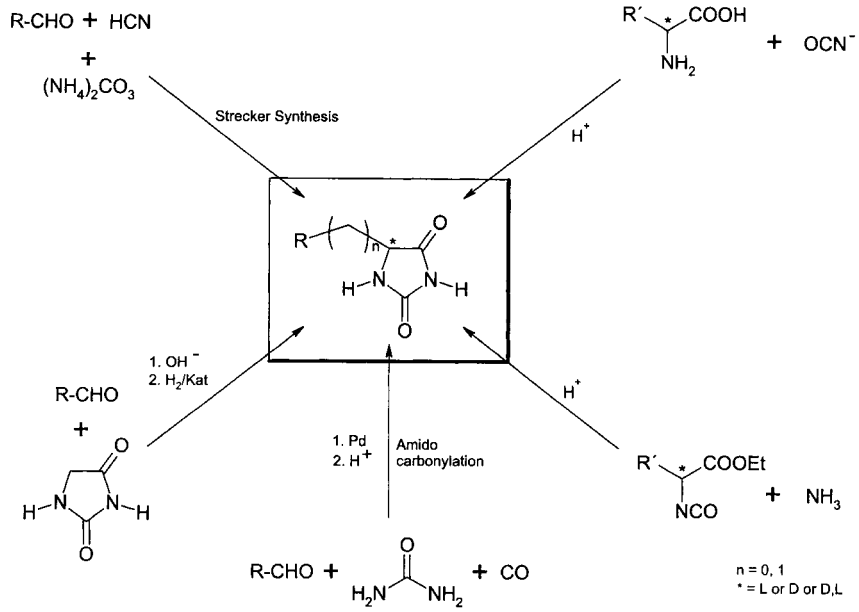


Figure 12.4-1. Chemical syntheses of hydantoins.

and[3], on their structures in solution, and in the solid state in reference[4]. With the increasing interest in new amino acid derivatives, recent investigations on their chemical synthesis concentrates on the development of "one-pot-syntheses" of the corresponding hydantoin derivatives, e.g. by carbonylation of aldehydes in presence of urea derivatives[5].

Many of the hydantoin derivatives are substrates for enzymatic reactions. It has been known since the 1940s that some microorganisms are able to grow on D,L-5-monosubstituted hydantoins as the sole C- and/or N-source in a mineral salt medium, often hydrolyzing only one enantiomer of a racemic mixture, and that even enzymes from plant and animal sources are able to hydrolyze and close the hydantoin ring. Various enzymes, so called hydantoinases, facilitate the hydrolysis of the hydantoin ring system in an initial reaction step. The biosynthesis of these enzymes often has to be induced by adding specific compounds during the growth of the microorganisms. The so-formed hydantoinases may have different substrate specificities and in general are selective in forming L- or D-*N*-carbamoyl amino acids (= hydantoic acids). The hydantoinases can often be found in combination with highly stereoselective *N*-carbamoylamino acid amidohydrolases (*N*-carbamoylases), which catalyze the further hydrolysis of the hydantoic acids to the free amino acids in an irreversible reaction. In some cases a hydantoin-racemase is involved as a third enzyme. Together, these three enzymes accomplish the total conversion of racemic D,L-5-monosubstituted hydantoin derivatives into the corresponding enantiomerically pure D- or L-amino acids. This cascade of reactions, whether located in whole cells or carried out using isolated enzymes is called the "hydantoinase-process".

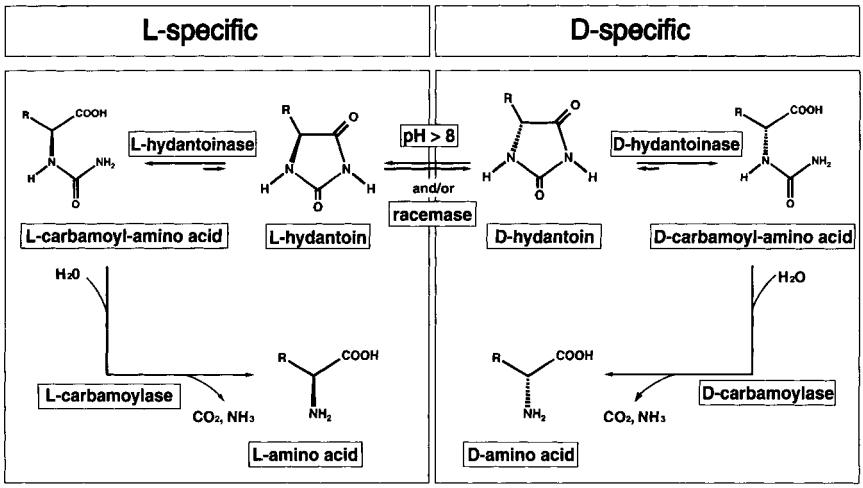


Figure 12.4-2. Reaction scheme for the enzymatic cleavage of D,L-5-monosubstituted hydantoin derivatives to the corresponding D- or L-amino acids.

Figure 12.4-2 shows the general reaction scheme for the enzymatic cleavage of D,L-5-monosubstituted hydantoin derivatives to the corresponding D- or L-amino acids.

The great advantages for industrial use of the hydantoinase-process are based on the fact that potentially 100% conversion and a 100% optically pure amino acid can be obtained at the same time if a racemic substrate is used. Until the mid 1990s in most cases, wild type strains, resulting from traditional screening methods (for a review see: reference [6]), were used as whole cell biocatalysts. Detailed reviews on the use of free or immobilized whole cell systems for hydantoin cleavage were given in references [3, 7, 8]. More recent activities are summarized in this chapter and concentrate on the use of recombinant free or immobilized enzymes (see Sect. 12.4.2-12.4.6), fusion proteins (see Sect. 12.4.7), specially designed recombinant whole cell biocatalysts (see Sect. 12.4.4) or the optimization of enzyme properties by directed evolution (see Sect. 12.4.7).

The hydantoinases belong to the E.C. 3.5.2 group of cyclic amidases [9], which is shown in Table 12.4-1. Of this group, four enzymes are original hydantoinases, because their substrates are naturally occurring hydantoin derivatives: carboxymethylhydantoinase (E.C. 3.5.2.4), allantoinase (E.C. 3.5.2.5), 1-methylhydantoinase (E.C. 3.5.2.14), and carboxyethylhydantoinase. All other enzymes listed have natural occurring cyclic amides as substrates (e.g. barbiturate, 5,6-dihydrouracil, 5,6-dihydroorotate).

From recent investigations on DNA- and amino acid sequences of the different cyclic amidases and subsequent phylogenetic analyses, it is known today that most of these enzymes not only share a number of highly conserved regions and invariant amino acid residues [10], but form a protein superfamily and are the product of a divergent evolution [11]. Although most of them only share limited sequence homology (identity < 15%) and therefore are only distantly related, it can be shown:

Table 12.4-1. Hydantoinases and cyclic amidases[9].

Recommended name	Other names	Systematic name	E.C.-number
Barbiturase		barbiturate amidohydrolase	3.5.2.1
Dihydropyrimidinase	D-hydantoinase	5,6-dihydropyrimidine amidohydrolase	3.5.2.2
Dihydroorotase	carbamoylaspartic acid dehydrase	L-5,6-dihydro-orotate amidohydrolase	3.5.2.3
Carboxymethylhydantoinase		L-5-carboxymethylhydantoin amidohydrolase	3.5.2.4
Allantoinase		allantoin amidohydrolase	3.5.2.5
Penicillinase	β-lactamase, Cephalosporinase	penicillin amido-β-lactam hydrolase	3.5.2.6
Imidazolone propionase		4-imidazolone-5-propionate amidohydrolase	3.5.2.7
5-Oxoprolinase (ATP-hydrolyzing)	pyroglutamase	5-oxo-L-proline amidohydrolase	3.5.2.9
Creatininase		creatinine amidohydrolase	3.5.2.10
L-Lysine-lactamase			3.5.2.11
6-Aminohexanoate-cyclic dimer hydrolase			3.5.2.12
2,5-Dioxopiperazine hydrolase			3.5.2.13
1-Methylhydantoinase (ATP-hydrolyzing)		1-methylhydantoin amidohydrolase	3.5.2.14
Carboxyethylhydantoinase		L-5-carboxyethylhydantoin amidohydrolase	
Indolylmethylhydantoinase		5-indolylmethylhydantoin amidohydrolase	

1. that most of them are members of a broad set of amidases with similarities to ureases and build up into a protein superfamily[11, 12], whereas
2. the ATP-dependent hydantoinases (see Fig. 12.4-3) are not related, and
3. that they share a metal-binding motif consisting of conserved histidine residues, which seems to have an important role to play in structure and activity[10, 11, 13].

The differences in enantioselectivity, often used for the classification of hydantoinases based on their biotechnological value, therefore do not reflect the evolutionary relationship of the different hydantoinases, which are forming a more diverse group of enzymes than was assumed earlier (for more details see reviews : references[14] and[13]). This protein superfamily probably has its origin in the prebiotic conditions of the primitive earth, where *N*-carbamoyl-α-amino acids rather than free α-amino acids are supposed to be the first synthons for prebiotic peptides in the evolution today[15].

This section will have a detailed look at the occurrence of the different cyclic amides in nature and their physiological role in various metabolic pathways. Allantoin is widely distributed in nature and is an important metabolite in the degradation of purine nucleotides (see Fig. 12.4-4). Allantoin occurs in all organisms that do not have uric acid as the final product of their purine degradation pathways, and is the substrate for the enzyme allantoinase or 5-ureidohydantoinase (E.C.

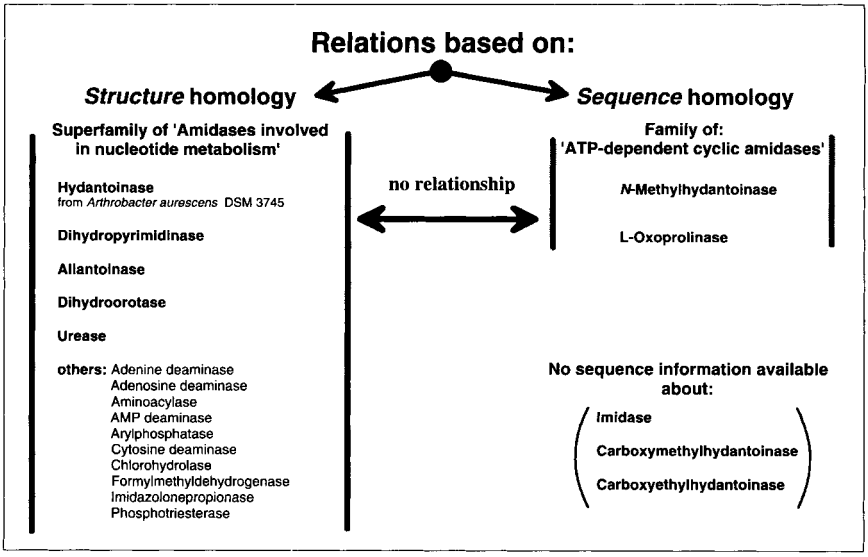


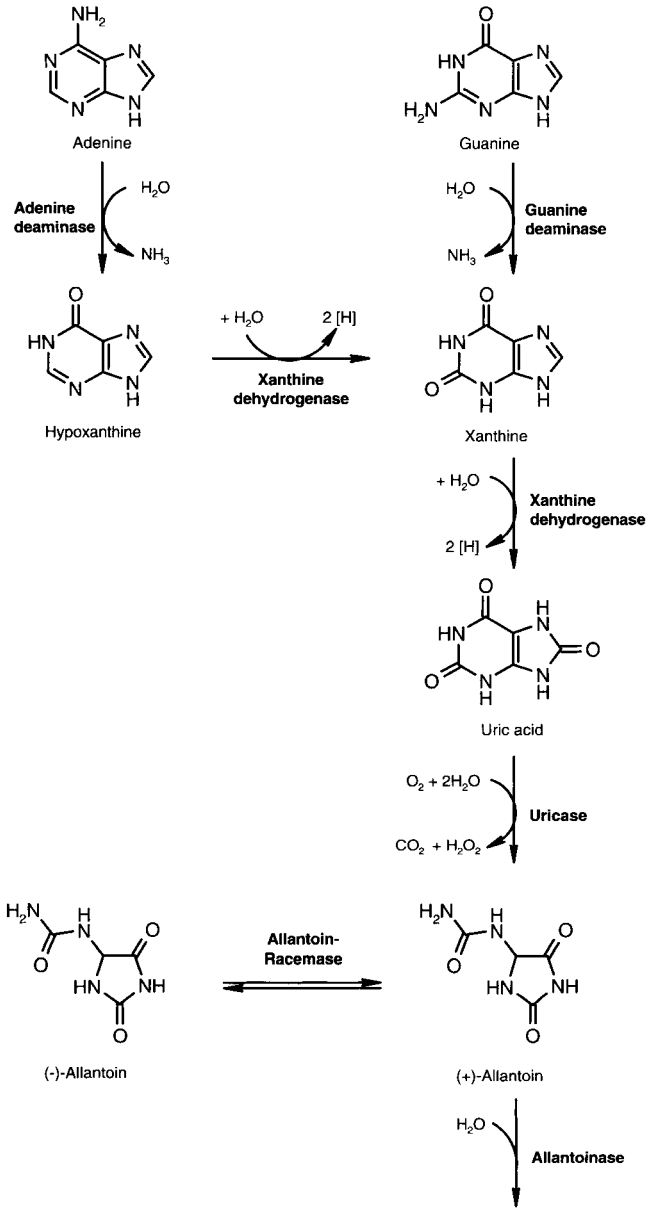
Figure 12.4-3. The evolutionary relationship of hydantoinases derived from sequence and structural similarity. Enzymes in bold letters are hydantoinases[13].

3.5.2.5), which can be found in microorganisms, plants and animals, either in combination with an allantoicase (E.C. 3.5.3.4) or an allantoate amidohydrolase (E.C. 3.5.3.9). The latter hydrolyzes allantoin to urea and glyoxylic acid, which are the final products of purine degradation in fishes. A recent paper describes the purification of this enzyme from *Bacillus fastidiosus*[16].

In the 1960s, different groups[17, 18] described the microbial enzyme as inducible and (+)-specific. Besides allantoin other inducers are compounds with a free ureido group such as *N*-carbamoyl-L-asparagine, *N*-carbamoyl-L-aspartate (the corresponding D-compounds were ineffective), hydantoate (i.e. *N*-carbamoylglycinate) and diureidomethane[19]. Information on the substrate specificity of allantoinases for other hydantoin derivatives is limited but D,L-5-aminohydantoin was shown to be accepted, albeit poorly, as a substrate[20]. Non-stereoselective allantoin hydrolysis and association of the allantoinase with a cofactor-independent allantoin racemase (E.C. 5.1.99.3) has been reported[20, 21], so that some microorganisms are also able to use (-)-allantoin as a substrate. An excellent review of these purine as well as pyrimidine degrading enzymes was given by Vogels and van der Drift[22].

The natural function of the carboxymethylhydantoinase (E.C. 3.5.2.2) is postulated to be the hydrolysis of 5-carboxymethylhydantoin, which is described to be the product of a non-enzymatic cyclization of *N*-carbamoyl-L-aspartic acid[23, 24] and to occur as a side-product in the metabolism of the pyrimidine nucleotide dihydroorotic acid[25]. This enzyme often occurs in combination with a ureidosuccinase (E.C. 3.5.1.7)[26], which catalyzes the cleavage of the resulting *N*-carbamoyl aspartic acid to L-aspartic acid (see Fig. 12.4-5). L-5-Carboxymethylhydantoin was first isolated after incubating orotic acid, a six-membered cyclic amide, with crude cell extracts of the anaerobic bacterium *Clostridium oroticum*[25, 26].

A third naturally occurring hydantoin, L-5-carboxyethylhydantoin, was first isolated by Brown and Kies[27] from the urine of rats, monkeys and humans after being fed ^{14}C-histidine, and it was postulated to be a by-product in the histidine degradation pathway shown in Fig. 12.4-6. Akamatsu[23] proved, by induction experiments, that the L-carboxyethylhydantoinase from a *Bacillus brevis* strain, also described by



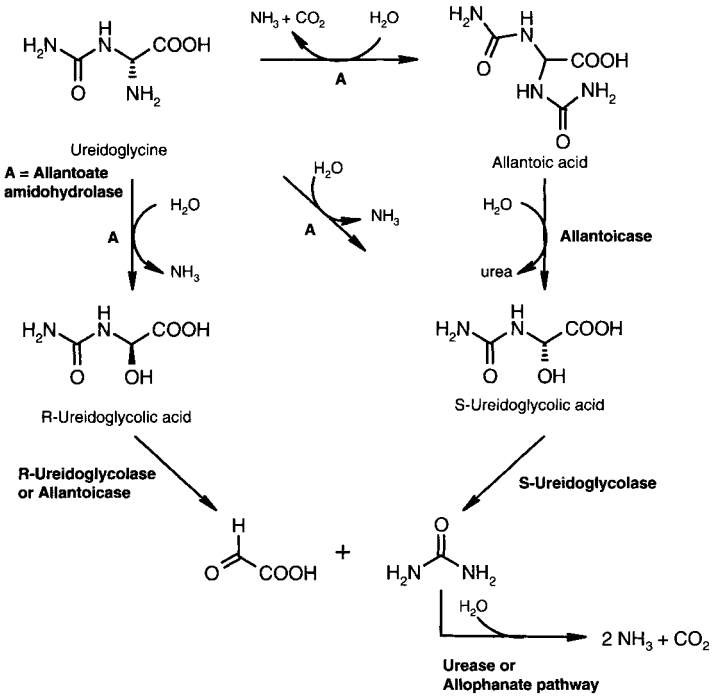


Figure 12.4-4. Purine degradation pathway via allantoin in microorganisms[22].

Tsugawa et al.[28] and Hassall and Greenberg[29] for the formation of L-glutamic acid from D,L-5-carboxyethylhydantoin, was not able to hydrolyze L-carboxymethylhydantoin and consequently it is not identical to the former enzyme described above. This enzyme has no E. C. number at present.

The six-membered ring systems 5,6-dihydropyrimidine, 5,6-dihydrouracil and 5,6-dihydrothymine can be hydrolyzed by the enzyme dihydropyrimidinase (E. C. 3.5.2.2), which is involved in the degradation of pyrimidine nucleotides. This widely spread, inducible catabolic enzyme is strictly D-selective in contrast to the L-selective dihydroorotase (E. C. 3.5.2.3), which is involved in the opposite anabolic pathway (see above). Another name often used in the literature for the dihydropyrimidinase is D-hydantoinase, because it is also able to hydrolyze D,L-5-monosubstituted hydantoin derivatives with high activity. Both reactions are shown in Fig. 12.4-7.

Natural cyclic amides such as 5,6-dihydrouracil, uracil and 5,6-dihydrothymine as well as hydantoin, 5-methylhydantoin and 5-hydroxymethylhydantoin are effective inducers for enzyme biosynthesis (for a more detailed review on induction experiments see reference[3]). In some cases, the dihydropyrimidinase (D-hydantoinase) is associated with an *N*-carbamoyl-D-amino acid amidohydrolase (D-carbamoylase) and a hydantoin racemase[30]. The previously proposed identity of the D-*N*-carbamoylase with the β-ureidopropionase (E. C. 3.5.1.6), which was assumed to be responsible for the hydrolysis of *N*-carbamoyl-β-alanine (see Fig. 12.4-7)[31–35] is no longer valid since the investigations of Ogawa et al. on different aerobic bacteria showed that the

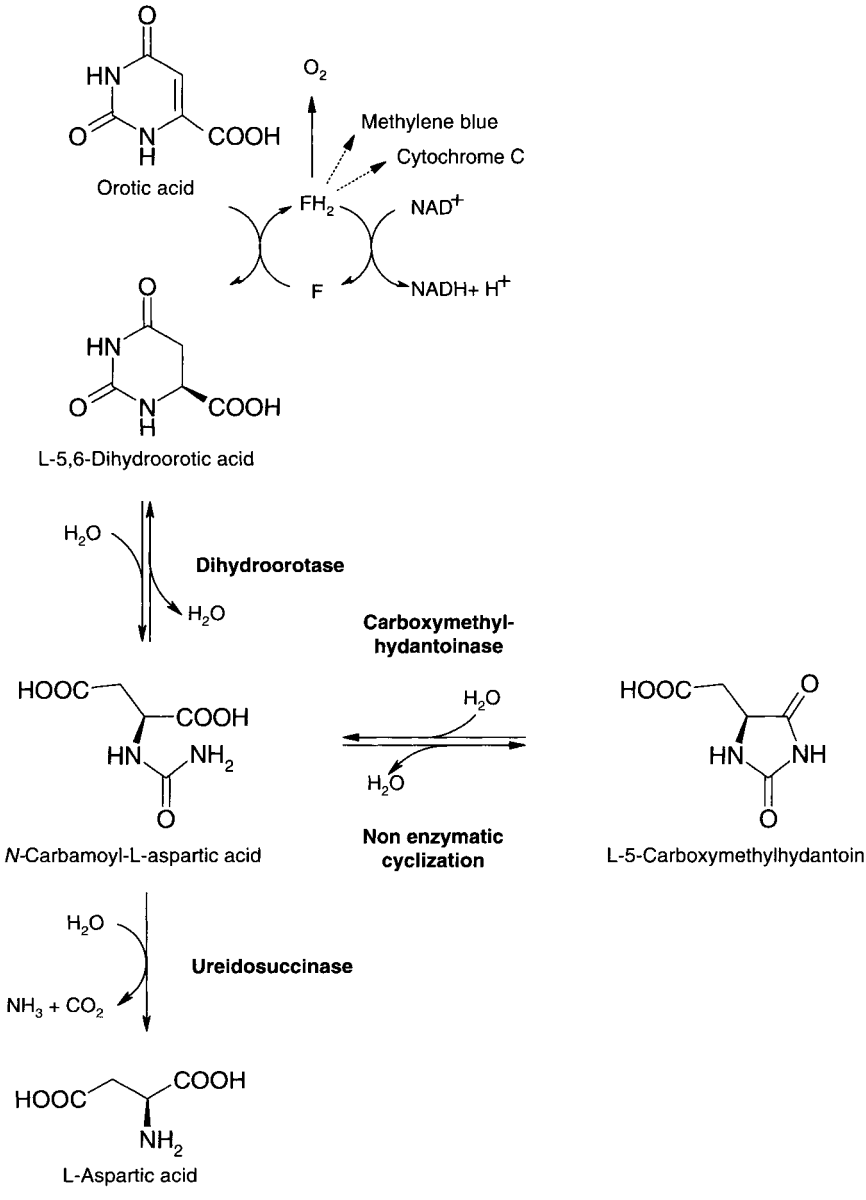


Figure 12.4-5. Metabolism of orotic acid and dihydroorotic acid[22, 24].

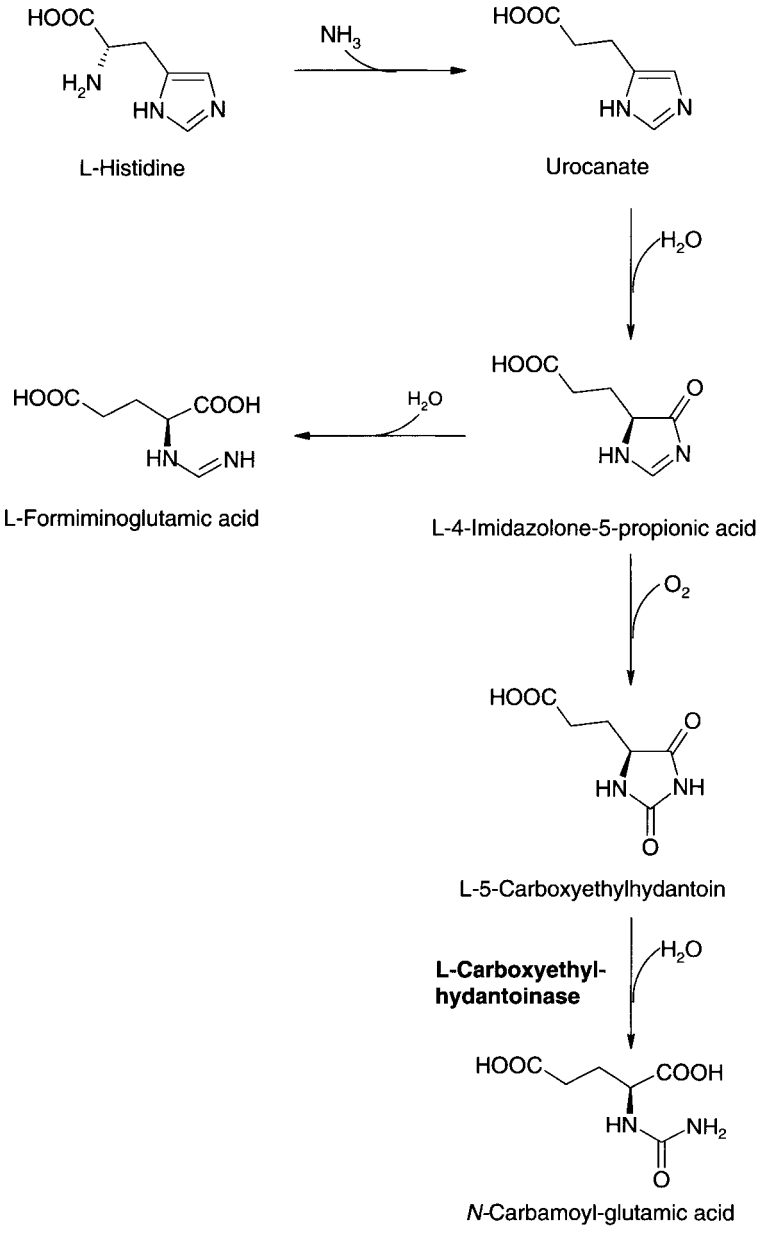


Figure 12.4-6. Histidine degradation pathway and carboxyethylhydantoinase-catalyzed reaction.

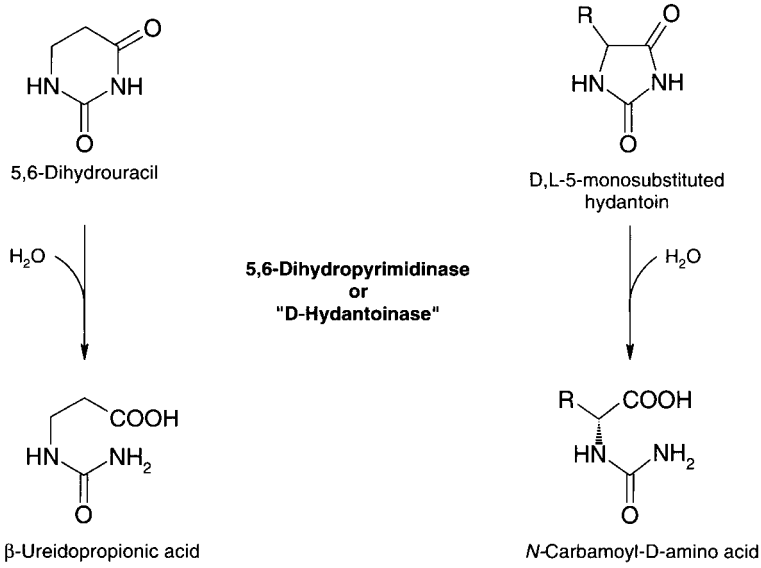


Figure 12.4-7. Analogy between dihydropyrimidinase- and D-hydantoinase-catalyzed reactions.

L-specific carbamoylase from *Pseudomonas putida* IFO 12996 also hydrolyzes β-ureidopropionate[14, 36]. The enzyme from *Pseudomonas putida* IFO 12996 was shown to be strictly L-selective and to be active on L-*N*-formyl- and also on L-*N*-acetyl-alanine[36]. In this context it may be of interest that Runser and Meyer described a D-hydantoinase with no dihydropyrimidinase activity[37] and Ogawa et al. reported on the occurrence of a D-*N*-carbamoylase with no relation to a D-hydantoinase[38].

Nevertheless, the dihydropyrimidinase seems to be closely related to the barbiturase (E.C. 3.5.2.1), which is able to hydrolyze barbituric acid[39] (Fig. 12.4-8).

The difference between barbituric acid and the natural compounds uracil and thymine is the presence of a keto-group instead of a methyl- or a hydrogen-group in the 6-position of the ring. Barbiturase was first detected by Hayashi and Kornberg[39] in bacteria of the genera *Mycobacterium* and *Corynebacterium* and postulated to catalyze a sidereaction in the degradation of pyrimidines. Unfortunately, there are no further data in literature on the substrate specificity and the stereoselectivity of this enzyme, which would allow comparison with the D-hydantoinase, but Kautz and

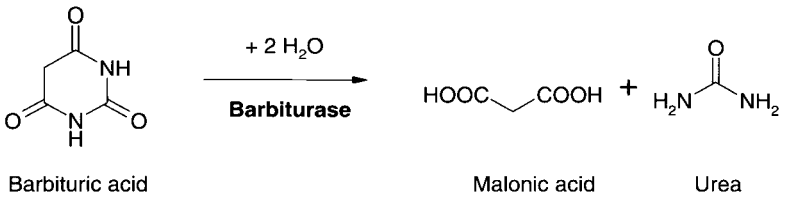


Figure 12.4-8. Barbiturase catalyzed reaction[39].

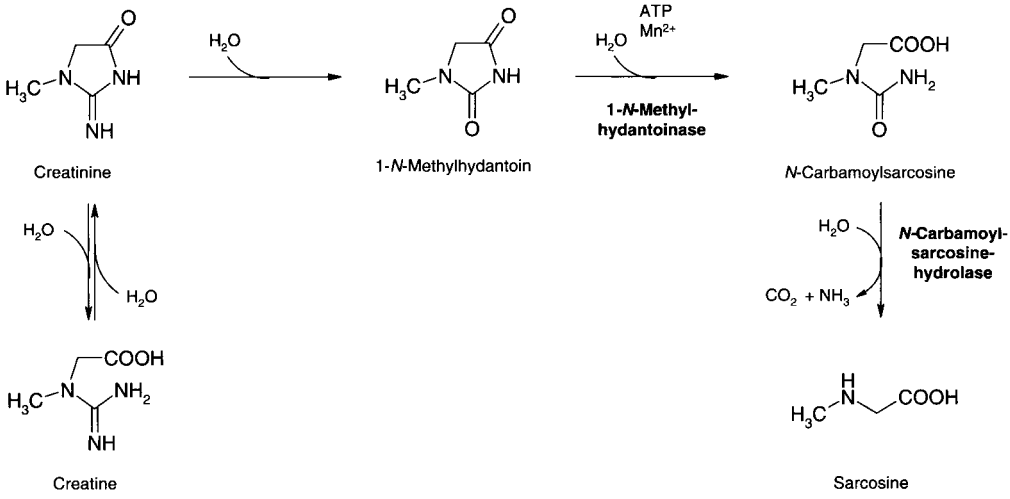


Figure 12.4-9. 1-Methylhydantoinase- and *N*-carbamoylsarcosine-amidohydrolase-catalyzed reactions in creatinine metabolism in bacteria.

Schnackerz were able to show that beef liver dihydropyrimidinase is also able to hydrolyze barbituric acid, although only with low activity[40].

Two other hydantoinases are described in the literature, which have not yet been listed in the Enzyme Nomenclature[9]. Siedel et al.[41], Yamada et al.[42, 43] and Ogawa et al.[44] found a new ATP-dependent 1-methylhydantoinase with additional nucleoside-triphosphatase activity[45] in different bacteria. This inducible enzyme, which was also shown to act on unsubstituted hydantoin and 5-methylhydantoin[41], is involved in the degradation of creatinine after its deimination in the 2-position to 1-methylhydantoin, resulting in *N*-carbamoylsarcosine (*N*-carbamoyl-*N*-methylglycine)[42, 43] (see Fig. 12.4-9). It is associated with a so-called D-*N*-carbamoylsarcosine hydrolase[43], which eventually hydrolyzes *N*-carbamoylsarcosine to free sarcosine. Both enzymes can be used for monitoring creatinine levels in blood[41].

Nishida et al.[46], Syldatk et al.[47, 48], Yamashiro et al.[49, 50], and Yokozeki et al.[51–53] found new L-5-arylalkylhydantoinases and a *N*-carbamoyl-L-amino acid amidohydrolases (L-*N*-carbamoylase), which are involved in the L-selective cleavage of 5-arylalkylhydantoins and could be most favorably induced by D,L-5-indolylmethylhydantoin or its *N*-3-methylated derivative[7]. The natural functions of these enzymes are not yet known, while one of the associated *N*-carbamoyl-L-amino acid amidohydrolases (L-*N*-carbamoylase) was also shown by Syldatk et al. to be reactive on *N*-formyl-L-amino acids[54]. In this strain both, hydantoinase and L-*N*-carbamoylase were shown to occur in combination with a hydantoin racemase[7, 55, 56]. Resting cells were used for the industrial production of L-amino acids from D,L-5-monosubstituted hydantoin derivatives as shown in Fig. 12.4-2[57].

Concerning their structure, cyclic imides are closely related to dihydropyrimidines and hydantoins. The metabolic transformation pathway for cyclic imides in microorganisms (see Fig. 12.4-10) was studied by Ogawa et al.[58, 59] in *Blastobacter* sp. and

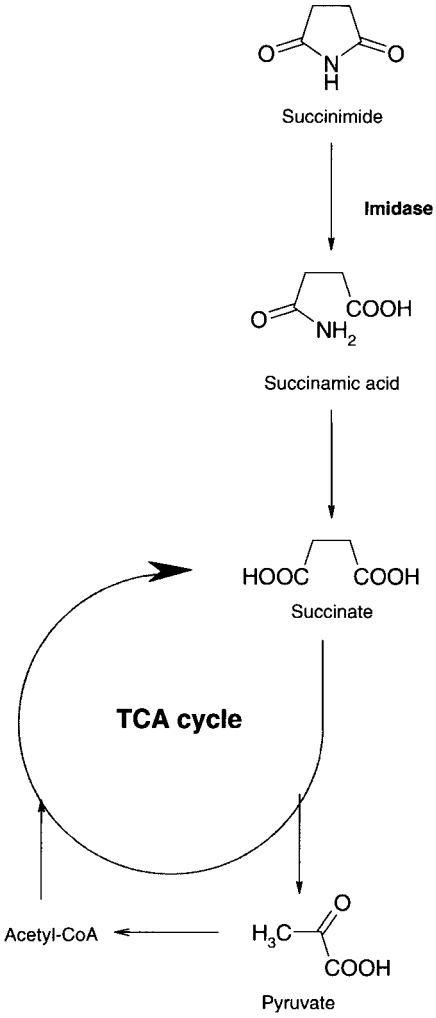


Figure 12.4-10. The proposed pathway for succinimide degradation in *Blastobacter* sp. A17P-4[59].

in different aerobic bacteria[60]. The enzyme involved in this reaction, a so called imidase, was also found to hydrolyze dihydropyrimidines[14].

Activity for the enzymatic cleavage of disubstituted hydantoins useful in the synthesis of α-,α-disubstituted amino acids was recently detected in crude enzyme extracts from the plant *Lens esculenta*[61, 62] and in papain by Rai and Taneja[63].

Of all the enzymes described above, at present only the D-hydantoinase- and the L-arylalkylhydantoinase processes are of significance for use in organic synthesis, in particular for the production of natural and non-natural optically pure D- and L-amino acids, and will be discussed in more detail in the following sections.

12.4.2
D-Hydantoinases – Substrate Specificity and Properties

Since the early 1950s it has been known that the inducible catabolic enzyme dihydropyrimidinase (E.C. 3.5.2.2) plays an important role in pyrimidine metabolism[23, 31, 33, 39, 64–66] and is widespread in nature. The natural substrates of this enzyme, which were also reported to be inducers, are 5,6-dihydrouracil and 5,6-dihydrothymine. Both compounds are important intermediates in the degradation of pyrimidine nucleotides. The dihydropyrimidinase-reaction is described to be strictly D-specific and to have a wide substrate specificity (see Fig. 12.4-11). In 1970 and

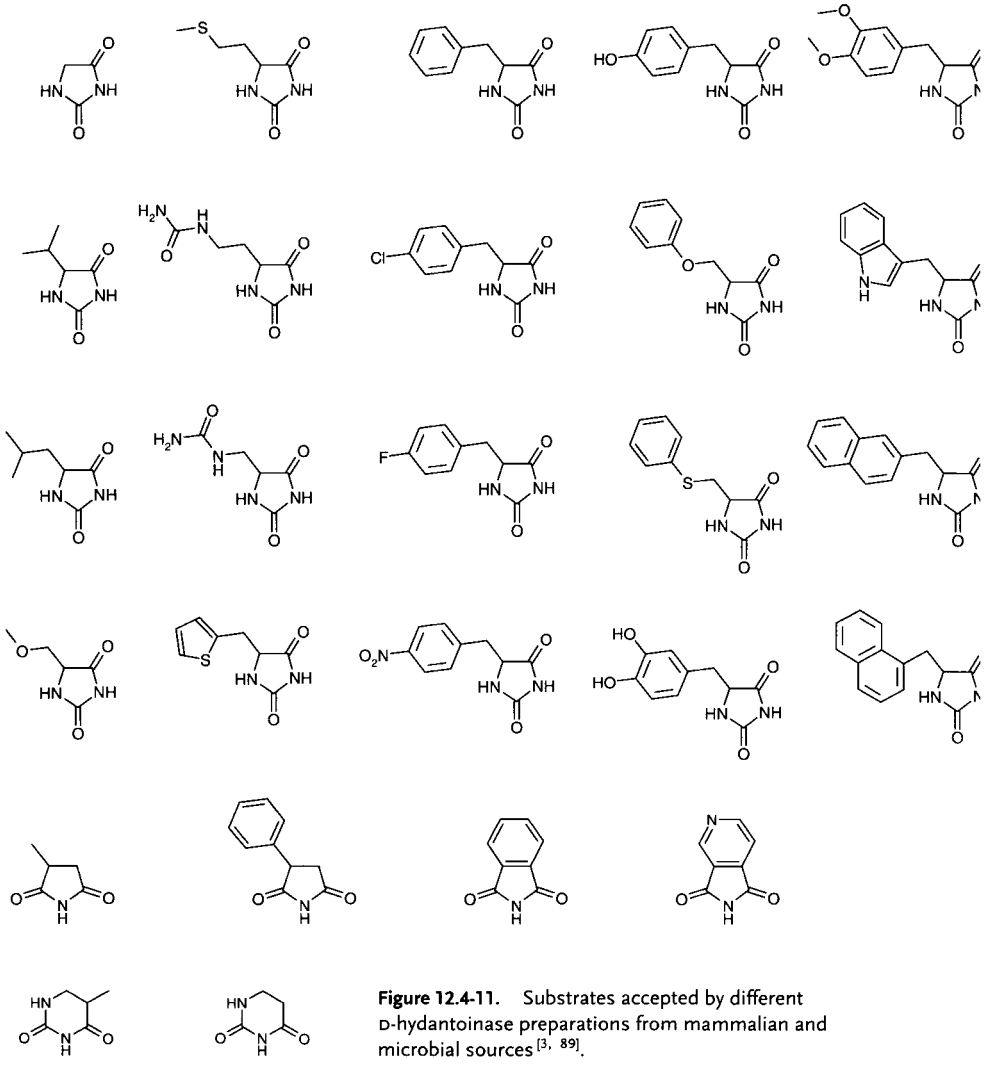


Figure 12.4-11. Substrates accepted by different D-hydantoinase preparations from mammalian and microbial sources[3, 89].

1973, Dudley et al. were the first to publish on the D-selective cleavage of 5-phenylhydantoin to *N*-carbamoyl-D-phenylglycine by a mammalian enzyme and on the spontaneous *in vivo* racemization of the residual L-isomer[67, 68]. In 1975, Cecere et al.[69] published on the enzymatic production of other *N*-carbamoyl-D-amino acids starting from chemically synthesized D,L-5-monosubstituted hydantoin derivatives using a partially purified fraction of the dihydropyrimidinase from calf liver. They were the first to stress that this enzyme might find an industrial application for the preparation of optically active D-amino acids as the so called "D-hydantoinase" (see Fig. 12.4-7). In 1978, the same group published on the production of various *N*-carbamoyl-D-amino acids using an immobilized calf liver dihydropyrimidinase preparation[70, 71]. Other publications have reported on the occurrence of D-hydantoinases in plant cell cultures[72]. Rai and Taneja published on the use of a plant enzyme from *Lens esculenta* immobilized to DEAE-cellulose for the same purpose[62].

In other publications, Wallach et al.[66], Brooks et al.[73] and Kautz and Schnackerz[40] gave detailed reports on the isolation and characterization of the dihydropyrimidinase from beef liver. Table 12.4-2 gives a short overview of the purification procedures and characteristic properties of these mammalian enzymes. The beef liver dihydropyrimidinase consists of four subunits and every active enzyme molecule contains four $Zn^{(II)}$cations[73] which are tightly bound ($K_S > 1.33 \times 10^9$ M^{-1}). In addition to 5,6-dihydrouracil, glutarimide, thiohydantoin and barbituric acid are also accepted as substrates, but with low reaction rates[40].

In the late 1970s the group of Yamada et al. in Japan postulated that in microorganisms the reason for the wide spread ability to hydrolyze D-selectively D,L-5-monosubstituted hydantoin derivatives was the existence of an enzyme called "D-hydantoinase"[74, 75]. With the increasing interest in the production of D-phenylglycine and D-*p*-OH-phenylglycine, since then several publications have described D-selective hydantoinases isolated from various microorganisms as *Pseudomonas striata*[75], *Pseudomonas fluorescens* DSM 84[76], *Pseudomonas* sp. AJ-11220[35], *Arthrobacter crystallopoietes* AM2[77], *Agrobacterium* sp. IP-I 671[37, 78], in anaerobic microorganisms[79], *Pseudomonas* sp. KBEL 101[80], *Agrobacterium tumefaciens*[81], thermophilic microorganisms[82], *Pseudomonas desmolyticum*[83], *Bacillus* sp.[84], *Bacillus stearothermophilus* SD-1[85, 86] and *Bacillus circulans*[87]. Runser and co-workers described a D-hydantoinase of an *Agrobacterium* sp. with remarkably high temperature and pH stability but no dihydropyrimidinase activity[37, 88]. Soong et al. were recently able to show that D-hydantoinase from *Blastobacter* sp.A17p-4 also is able to hydrolyze cyclic imides with bulky substituents to the corresponding half-amides and postulated that this enzyme may also function in cyclic imide metabolism in addition to pyrimidine metabolism[89]. New screening methods for isolation of D-hydantoinase-producing microorganisms were described by Morin et al. using a continuous cultivation system[90], and by LaPointe et al. using a polymerase-chain-reaction-amplified DNA probe to detect D-hydantoinase-producing microorganisms by direct colony hybridization[91].

A survey of the isolation and some characteristic data on some of the bacterial enzymes, which seem to be rather similar to the dihydropyrimidinases from mammalian tissues (Table 12.4-2) and plants, is given in Table 12.4-3.

Table 12.4-2. Purification and characteristic properties of D-hydantoinase from animal cells.

Source	Acetone powder from beef liver	Catalase fraction from beef liver	Acetone powder from beef liver
Reference	[66]	[73]	[40]
Purification steps	acid and heat treatment, ammonium sulfate and acetone precipitation	hydrophobic chromatography or preparative electrophoresis	heat treatment, ammonium sulfate precipitation, chromatography on chelating and DEAE-Sepharose
Yield (%)	25	13	44
Purification factor	200	24.2	186
Purity	80%	homogeneous	homogeneous
Optimal pH	8.2	no data given	8–10
Metal ion requirements	Mn^{2+} and Mg^{2+} (only when dihydrouracil is the substrate!)	Zn^{2+} and Co^{2+}	one Zn^{2+} per subunit
Molecular mass		226 000 Da	217 000 Da
Subunits		4×56 500 Da	4×54 000 Da

Figure 12.4-11 gives a survey of the substrates accepted by the different dihydropyrimidinase or D-hydantoinase preparations The differences between the enzyme preparations from mammalian and microbial sources are discussed in more detail in reference[3], but D-hydantoinases or dihydropyrimidinases, respectively, seem to have the following in common: (i) a wide substrate specificity, (ii) metal dependence and (iii) that they are strictly D-specific. Preferably, cyclic amides are hydrolyzed at pH values around 8.5. Furthermore, most of the enzymes are also described to be able to catalyze the hydantoin formation: the optimal pH of this reaction is neutral or weakly acidic.

In 1983 the first gene sequence of a D-hydantoinase derived from thermophilic *Bacillus* sp. LU 1220 and its overproduction in *Escherichia coli* HB 101 was published[92]. Not until 1994 were cloning, sequencing and expression of a D-hydantoinase gene from *Pseudomonas putida* DSM 84 in *Escherichia coli* reported[93], shortly followed by a paper on cloning, sequencing and expression of a thermostable D-hydantoinase from *Bacillus stearothermophilus* NS 1122A[94]. The same was described for the strain *Bacillus stearothermophilus* SD-1 by Lee et al. in 1997[95]. The same group reported that the *C*-terminal region of the D-hydantoinase was not essential for catalytic activity but affected the oligomeric structure of the enzyme[96]. In 1998, Chien et al. described the cloning, sequencing and expression of the D-hydantoinase gene from *Pseudomonas putida* CCRC 12857 in *Escherichia coli*[97]. Molecular cloning and sequencing of a cDNA encoding dihydropyrimidinase from rat liver was reported by Matsuda et al.[98], and the complete sequencing of a 24.6 kB segment of yeast chromosome XI including homologies to D-hydantoinases by Tzerma et al.[99].

D-Phenylglycine and D-*p*-OH-phenylglycine are important side chain moieties in the synthesis of semisynthetic penicillins and are produced in several thousand tons per year using the hydantoinase process[3, 100]. The different methods that this

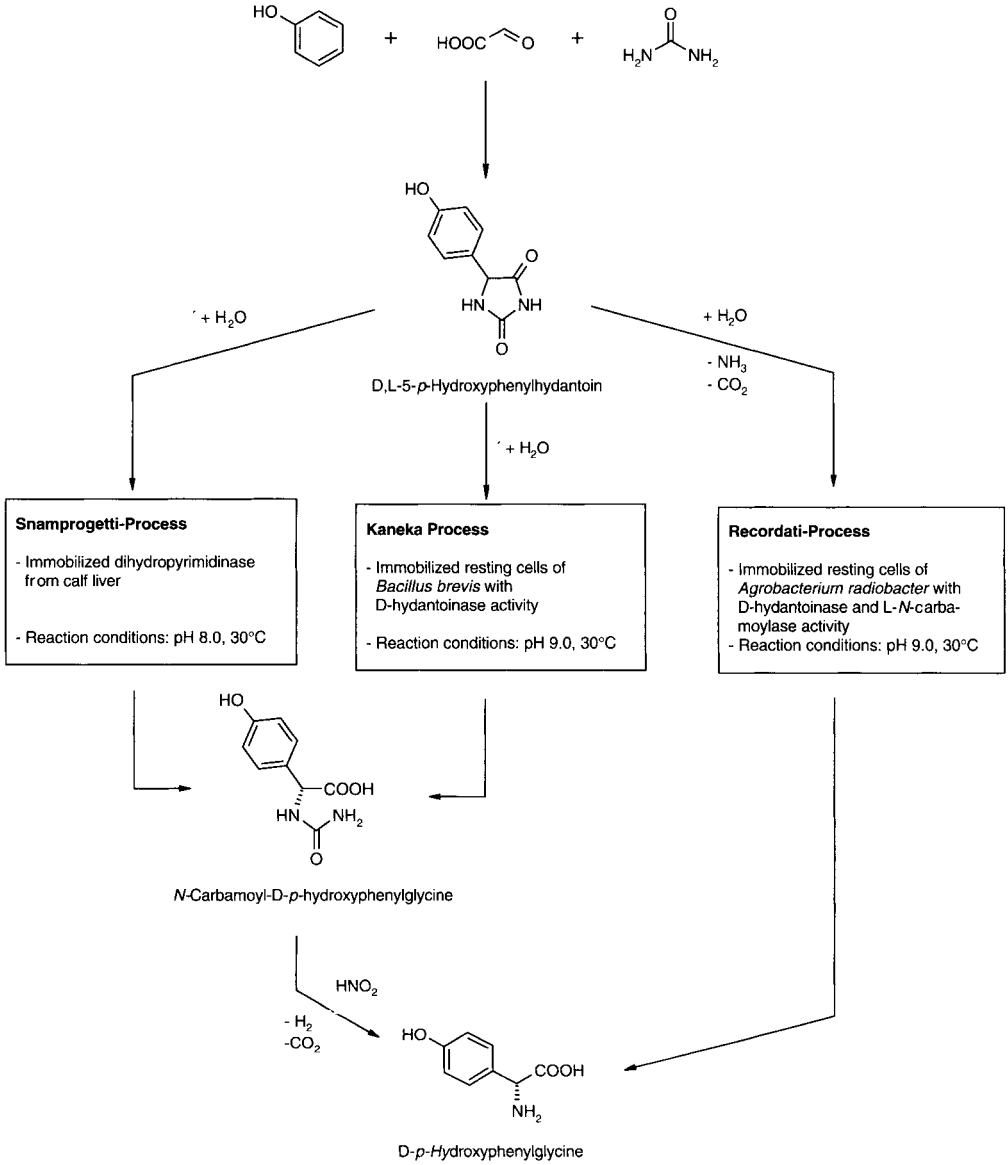


Figure 12.4-12. Industrial production of D-4-hydroxyphenylglycine acids by the D-hydantoinase process.

reaction has been realized in industrial application in recent years can be seen in Fig. 12.4-12.

In the 1970s, the company Snamprogetti first reported on the use of the beef liver dihydropyrimidinase immobilized on an ion exchanger for the continuous production of D-phenylglycine[70, 71], while the company Kanekafuchi was reported to use

resting cells of a *Bacillus* sp. containing D-hydantoinase activity only[100]. Because of missing D-*N*-carbamoylase activity or the instability of this enzyme in resting microbial cells, the decarbamoylation of the resulting D-*N*-carbamoylamino acid is often performed chemically by treatment with HNO_2. Because of the high stability of the D-hydantoinase it is possible to use immobilized resting cells, which can be applied repeatedly.

With the increasing interest in products other than D-phenylglycine and D-*p*-OH-phenylglycine, the companies Recordati and Degussa reported on the use of resting cells of an *Agrobacterium radiobacter* with high activities for both the D-hydantoinase and D-*N*-carbamoylase[100, 101]. The advantage of this process in comparison with the methods mentioned above is not only the environmental friendly "one pot production" of D-amino acids without use of HNO_2' but the possibility of also producing D-amino acids, which are unstable against treatment with this acid (e.g. D-tryptophan, D-citrulline or D-pyridylalanine) (for the production of D-citrulline from L-ornithine see Fig. 12.4-13).

Nevertheless, the main problem of using resting cells in a "one pot process" still seems to be the stability of the D-*N*-carbamoylase (see e.g. reference[80]), which is discussed in Sect. 12.4.3. Therefore, a series of papers from the 1990s concentrated on: the optimization of the chemoenzymatic D-hydantoinase catalyzed production of D-*N*-carbamoylphenylglycine and D-*N*-carbamoyl-4-hydroxy-OH-phenylglycine as the enhanced chemical decarbamoylation of D-*N*-carbamoylphenylglycine by its interfacial solubilization under micellar conditions; the repeated use of a commercially available covalently immobilized D-hydantoinase at high substrate concentrations[102], the repeated use of a thermostable D-hydantoinase from *Bacillus stearothermophilus* SD-1 immobilized on DEAE-cellulose resin[103], the mass production of the same enzyme in *Escherichia coli* using a constitutive expression system[95]; the application of numerical modeling for optimization of a complex medium for D-hydantoinase production from *Agrobacterium radiobacter* NRRL B 11291[104]; the modeling, simulation and kinetic analysis of a heterogeneous reaction system for the conversion of D,L-4-hydroxy-phenylglycine to the corresponding D-*N*-carbamoyl amino acid[105], the use of a so called "pressure swing reactor" for the same purpose[106] as well as on the racemization of the remaining substrate enantiomers[107].

12.4.3
D-*N*-Carbamoylases – Substrate Specificity and Properties

In some cases, D-hydantoinases are described as being associated strictly with D-specific *N*-carbamoyl-D-amino acid amidohydrolases (D-*N*-carbamoylases). One natural role of these enzymes was discussed as being the β-ureidopropionase (E.C. 3.5.1.6), which catalyzes the decarbamoylation of β-ureido propionic acid in pyrimidine metabolism (see Fig. 12.4-7), but with the recent information on its stereoselectivity[36] and its DNA and amino acid sequences, this previously proposed homology[31–35] is no longer clear.

Various D-*N*-carbamoylases were purified from rat liver as well as from microbial

Table 12.4-3a. Purification and characteristic properties of microbial D- and L-hydantoinases.

Source	*Arthrobacter crystallopoietes* DSM 20117	*Arthrobacter aurescens* DSM 3745	*Pseudomonas fluorescens*	*Pseudomonas striata*	*Pseudomonas* sp. AJ 11220
Reference	[77, 159]	[126, 127, 128, 130]	[76]	[75]	[35]
Inductor	dihydrouracil, hydantoin and various D,L-5-monosubstituted hydantoins	*N*-3-Methyl-D,L-5-indolylmethylhydantoin		Hydantoin	5-cyanoethyl-hydantoin
Purification	protamine sulfate and ammonium sulfate precipitation, IEX (DEAE-cellulose), HIC (Phenylsepharose), Mono Q, gel filtration	DEAE-Streamline, HIC (Phenylsepharose), Mono Q	HIC (Phenylsepharose), SEC (Sephacryl S-400) and preparative electrophoresis	protamine sulfate and ammonium sulfate precipitation, IEX (DEAE-cellulose), hydroxyl apatite- and SEC (Sephadex G 200), crystallization	IEX (DEAE-Toyopearl)
Yield (%)	5	77	1	3	63
Purification factor	20	52.6	0.53	300	27
Purity	homogenous	homogeneous	homogeneous	homogeneous	crude enzyme
Optimal temperature (°C)	50–60	50	55	45–55	55
Temperature stability (°C)	<50		<40	<60	
Optimal pH	8.2–9.2	8.8–9.3	9.0	8.0–9.0	8.0
pH stability	6.5		5.5–8.5	6.0–7.0	
Metal ion requirements	Zn^{2+}	10 mol Zn^{2+} per mol of active enzyme, but Mn^{2+} and Co^{2+} Enhance the enzymatic activity	Fe^{2+}	Fe^{2+}, Co^{2+}	
Molecular mass (Da)	257 000	200 000	230 000	190 000	
Subunits (Da)	4×60 000	4×49 680	4×60 000		

Table 12.4-3b. Purification and characteristic properties of microbial D- and L-hydantoinases.

Source	*Bacillus stearothermophilus* SD-1	*Bacillus circulans*	*Blastobacter* sp. A17p-4	*Agrobacterium* sp. IP-I 671
Reference	[85, 86]	[87]	[89, 160]	[37, 88]
Inductor	hydantoin	methylthioethylhydantoin	uracil	uracil
Purification	ammonium sulfate fractionation, Q-Sepharose, heat treatment, HIC (phenylsepharose, preparative gel electrophoresis)	heat treatment, Sephadex G-50, DEAE-cellulose, HIC (phenylsepharose), Fractogel	DEAE-Sephacel, HIC (phenylsepharose), SEC (Sephacryl S-200 HR), Mono Q and superose-12	protamine sulfate and ammonium sulfate precipitation, heat treatment, IEX (DEAE-Sephadex and Trisacyl, HIC (octyl-Sepharose)
Yield (%)	1.5	12.4	3	9
Purification factor	50	243	30	965
Purity	homogenous	homogeneous	homogeneous	homogeneous
Optimal temperature (°C)	65	75	60	60
Temperature stability (°C)	<60	<60	<60	< 70
Optimal pH	8.0	8.0–10.0	10.0	10.0
pH stability	5.5–11.0	8.5–9.5	5.0–10.0	7.5–10.5
Metal ion requirements	Mn^{2+}	Mn^{2+}, Co^{2+}, Ni^{2+}		
enhance the enzymatic activity	Mg^{2+}, Mn^{2+}, Co^{2+}, Ni^{2+}			
enhance the enzymatic activity	Ni^{2+}, Mg^{2+}			
Molecular mass (Da)	126 000	212 000	200 000	250 000
Subunits (Da)	2×54.000	4×53 000	4×53 000	4×62 000

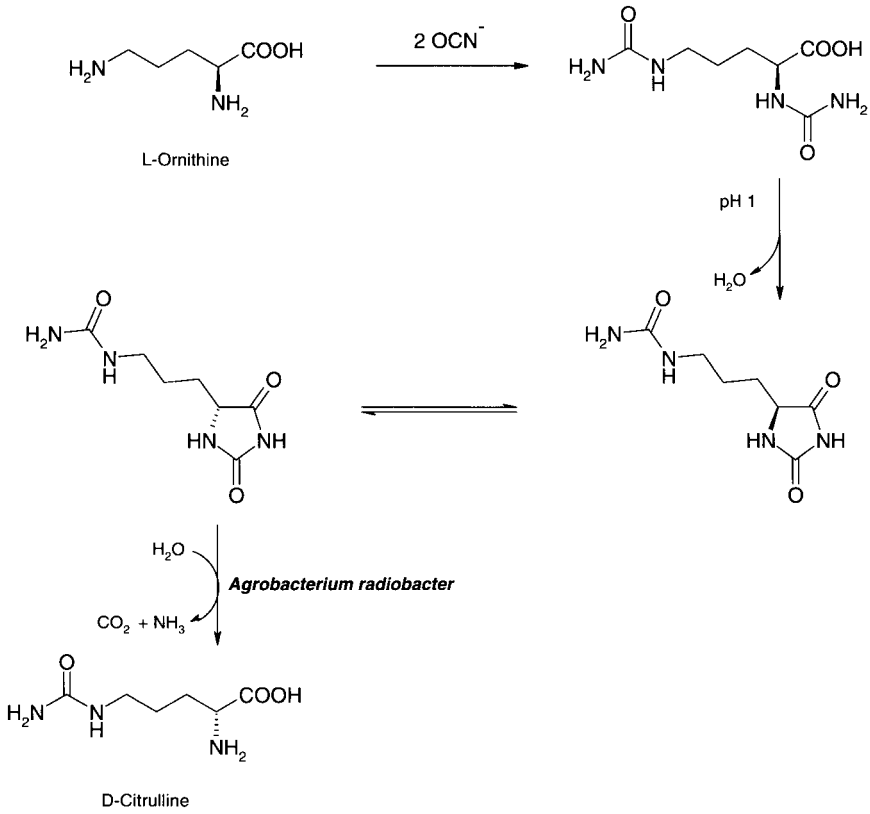


Figure 12.4-13. Production of D-citrulline from L-ornithine by means of *Agrobacterium radiobacter.*

cells. In contrast to D-hydantoinases (see above), induction and stability of these enzymes seem to be problematic[3]. Meyer and Runser reported that both D-hydantoinase and D-*N*-carbamoylase were found to be highly inducible by the addition of non-metabolizable thiolated hydantoins or pyrimidines to the culture medium of *Agrobacterium* sp. I-671[108].

Rat liver D-*N*-carbamoylase was isolated by Caravaca and Grisolia[31]. The same authors also found it in the supernatants of liver homogenates of dogs, pigeons and rabbits. In microorganisms, D-*N*-carbamoylase activity was detected in various strains of *Agrobacterium* sp.[32, 80, 109–111], *Blastobacter* sp. A17p-4[112], *Clostridium uracilicum*[33], *Comamonas acidovorans*[38], *Pseudomonas putida* 77[43] and *Pseudomonas* sp. AJ-11220[75]. Induction of enzymatic activity during growth was done either by addition of *N*-carbamoyl amino acids or pyrimidine and hydantoin derivatives. Some of the enzymes were purified and characterized as shown in Table 12.4-4.

The enzyme isolated from rat liver and the inducible *Clostridium* D-*N*-carbamoylase are both postulated to be involved in the degradation of pyrimidines[33]. With only a few compounds having been tested as substrates for these enzymes, they are

Table 12.4-4a. Purification protocols and characteristic properties of microbial D-*N*-carbamoylases.

Source	*Agrobacterium radiobacter* NRRL B11291	*Agrobacterium* sp. KNK712 (after expression in *Escherichia coli*)	*Agrobacterium* sp. (BEECHAM-strain)	*Clostridium uracilicum*	*Comamonas* sp. E 222c
Reference	[113, 116]	[111]	[110]	[33]	[38]
Inductor	*N*-carbamoyl-D-phenylglycine	urea, *N*-carbamoyl-phenylglycine, *N*-carbamoyl-phenylglycine (for the wild strain)	strain was genetically engineered – no data are given on this	*N*-carbamoyl-β-alanine	*N*-carbamoyl-β-alanine
Purification	Q-Sepharose FF, chelating Sepharose, Superose 12 (procedure for the recombinant enzyme)	heat treatment, HIC (Phenylsepharose), ammonium sulfate precipitation, DEAE-Sepharose (data for the recombinant enzyme)	clarified crude extract was used for experiments	$MnCl_2$, ammonium sulfate and acetone precipitation, hydroxyl apatite chromatography	ammonium sulfate precipitation, IEX (DEAE-Sephacel, MonoQ), HIC (Phenylsepharose)
Yield (%)	34 (for the recombinant enzyme)	12.3 (data for recombinant enzyme)	clarified crude extract	18	36
Purification factor	20 (for the recombinant enzyme)	3.9 (data for recombinant enzyme)	clarified crude extract		119
Purity		homogenous	clarified crude extract	crude enzyme	homogeneous
Optimal temperature (°C)	60	65	52	30–35	40 °C
Temperature stability	< 40 °C	< 55 °C		< 45 °C	< 40
Optimal pH	7.0	7.0	7.4–7.6	7.4–7.8	8.0–9.0
pH stability	7.0–9.0	7.0–9.0	6.2–9.0		6.5–9.5
Metal ion requirements	none	no details given	none	none	none
Molecular mass (Da)	68 000	no details given	84 000 (determined by native gel filtration)	no details given	111 000
Subunits (Da)	34 000	34 285 (calculated)	no details given	no details given	3×40 000

Table 12.4-4b. Purification protocols and characteristic properties of microbial D-*N*-carbamoylases.

Source	*Blastobacter* sp. A17p-4	*Pseudomonas* sp. AJ-11220	*Pseudomonas putida* 77
Reference	[112]	[35]	[43]
Inductor	uracil	5-cyanoethylhydantoin	1-methylhydantoin
Purification	ammonium sulfate precipitation, DEAE-Sephacel, HIC (Phenylsepharose), Sephadex G150, Mono Q	IEX (DEAE Toyopearl)	ammonium sulfate precipitation, IEX (DEAE-Cellulose), crystallization
Yield (%)	2.3	36	63.2
Purification factor	37	17	27.4
Purity	homogeneous	crude enzyme	homogeneous
Optimal temperature (°C)	55	55	37
Temperature stability (°C)	< 50		< 40
Optimal pH	8.0–9.0	7.0	7.0–8.0
pH stability	6.0–9.0		6.0–7.0
Metal ion requirements	no details given	no details given	no details given
Molecular mass (Da)	120 0000	no details given	102 000
Subunits (Da)	3×40 000	no details given	4×27 000

obviously different from the other microbial D-*N*-carbamoylases listed in Table 12.4-4.

The *N*-carbamoylsarcosine amidohydrolase from *Pseudomonas putida* 77 is reported to have its biological function in creatinine metabolism[43]. The D-*N*-carbamoylases of the various *Agrobacterium* sp. and the *Pseudomonas* sp. AJ-11220 are likely to be identical. They have a wide substrate specificity in common, for a survey see Fig. 12.4-14, and hydrolyze only the D-enantiomers of aliphatic and aromatic hydantoic acids[35, 113]. The main problems of this enzyme seem to be (i) its instability and its rapid inactivation in absence of a reducing agent[110] probably caused by an oxidation of an SH-group[114] and (ii) its inhibition by ammonium ions[115]. Grifantini et al. were able to prove the role of the cysteine 172 out of five cysteines for enzyme activity by site-directed mutagenesis[116], while Nanba et al. were able to obtain a more thermotolerant D-*N*-carbamoylase by substitution of Pro 203 by Leu in the gene from *Agrobacterium* sp. KNK712 before expression in *Escherichia coli*[111]. For stabilization, the same group immobilized the enzyme by glutaraldehyde coupling to Duolite A-568, a macroporous phenol formaldehyde resin[114]. Kim and Kim tried to overcome limitations in the production of D-p-OH-phenylglycine with resting cells of *Agrobacterium* sp. I-671 by adsorptive removal of the ammonium ions with a silicate complex[115] and proposed the optimized ratio between D-hydantoinase and D-carbamoylase of about 1 : 3 based on mass for further process optimization[117].

As discussed before, there is a lot of interest in microbial biocatalysts with highly active D-hydantoinase- and D-*N*-carbamoylase-activity for the direct synthesis of HNO_2-sensitive D-amino acids used as chiral synthons in the production of pharma-

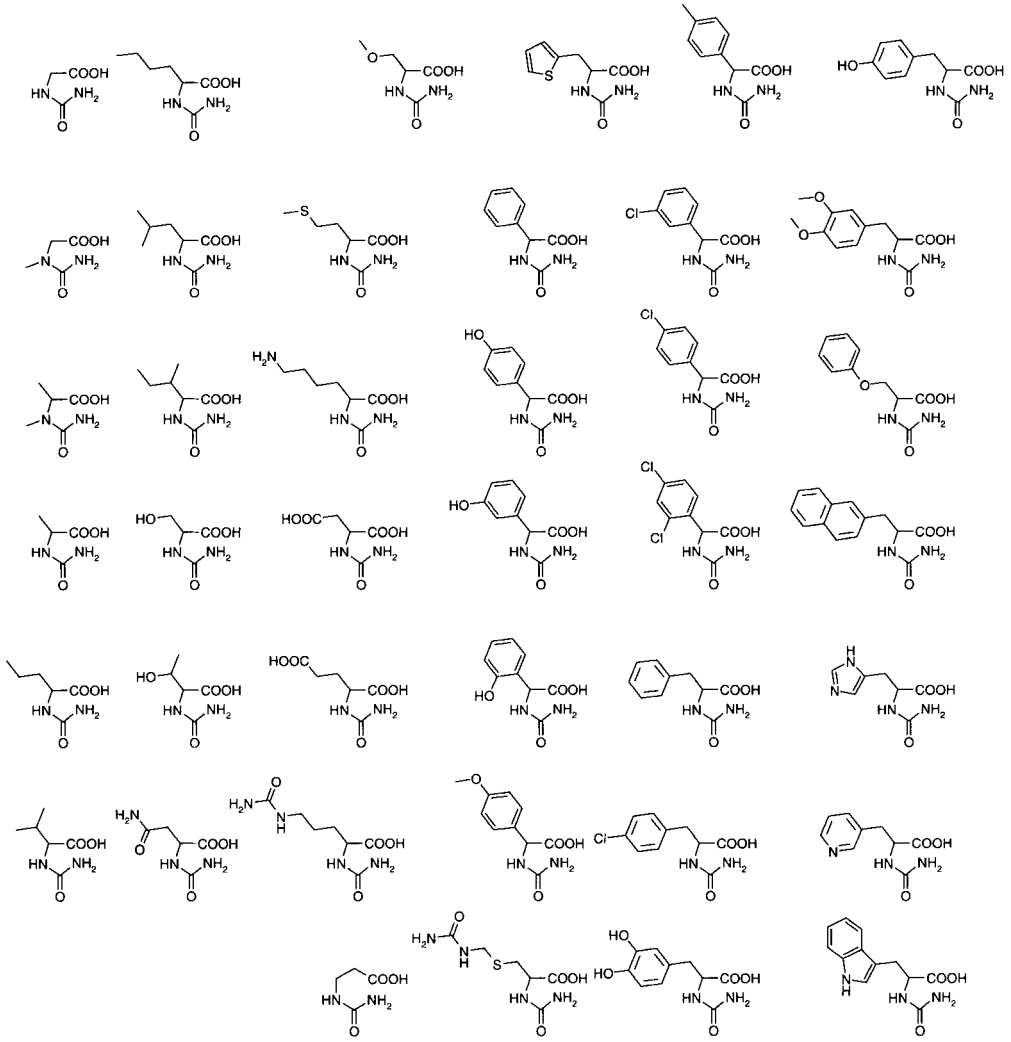


Figure 12.4-14. Substrates accepted by microbial D-*N*-carbamoylases of *Agrobacterium radiobacter* and *Pseudomonas* sp. AJ-11220[35, 113].

ceutical drugs and intermediates. For the synthesis of peptides in particular, a great variety of D-amino acids and derivatives are highly desirable molecules. Recently, cell free extracts of *Blastobacter* sp. A17p-4 were used for the preparation of optically active D-*p*-trimethylsilylalanine from the corresponding D,L-carbamoyl amino acid[118] and several biocatalysts (isolated enzymes as well as whole cells) have been compared with respect to stereoselectivity for the hydrolysis of D,L-5-trimethylsilylhydantoin[119]. Cell free extracts of *Blastobacter* sp. A17p-4 were shown to distinguish stereoisomers of hydantoins not only at the α-carbon but also at the β-carbon of *N*-carbamoyl-α,β-amino acids[120].

12.4.4
L-Hydantoinases – Substrate Specificity and Properties

In the 1960s, Tsugawa et al.[28] were able to isolate strains of *Pseudomonas, Micrococcus, Aerobacter, Achromobacter,* and *Bacillus* that were capable of producing L-glutamic acid from D,L-5-carboxyethylhydantoin by L-5-carboxyethylhydantoinase. *Bacillus brevis* ATCC 8185 was the first microorganism used for bioconversion of a racemic hydantoin derivative to an L-amino acid in the case of L-glutamic acid with a yield of 90%. In 1988, Yamashiro et al.[49, 50] reported on an L-hydantoinase from *Bacillus brevis* AJ 12299. This *Bacillus* L-hydantoinase requires ATP and Mg^{2+}, Mn^{2+} or K^+ as cofactors and acts selectively on L-configured substrates. The optimal reaction conditions for the hydantoin cleavage are pH 8.0 and 50 °C. Only a few substrates have been investigated as shown in Fig. 12.4-15, so it is not clear whether this enzyme may be identical to the L-5-carboxyethylhydantoinase described before (see Sect. 12.4.1).

Watabe et al. reported on the cloning and sequencing of genes for an L-hydantoinase deriving from *Pseudomonas* sp. NS 671 able to convert L-selective D,L-5-MTEH, a precursor of methionine[121]. Production of L-methionine from the corresponding hydantoin derivative was also described by Ishikawa et al. for resting cells of *Bacillus stearothermophilus* NS1122A[122] after growth of this strain on a medium containing D,L-5-MTEH as an inducer. The resting cells were described to be stimulated by addition of cobalt and manganese ions, while copper and zinc ions caused a strong inhibition of the enzymatic activities. Wagner et al. described the use of an *Arthrobacter* sp. DSM 7330 for the production of L-methionine and were able to obtain product concentrations of up to 120g L^{-1} using a special feed-batch technique for feeding of the hydantoin substrate[123].

From the data available, the three L-hydantoinases from *Bacillus brevis* and *Bacillus stearothermophilus* and the enzyme from *Pseudomonas* mentioned above seem to have a preference for hydantoin derivatives containing aliphatic side chains and therefore differ distinctly from those enzymes found in *Arthrobacter* sp. by Cotoras et al.[124],

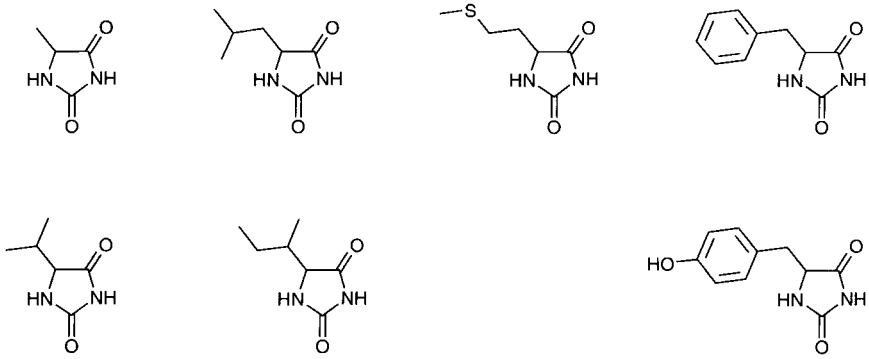


Figure 12.4-15. Substrates accepted by the L-hydantoinase of *Bacillus brevis* AJ 12299[49, 50].

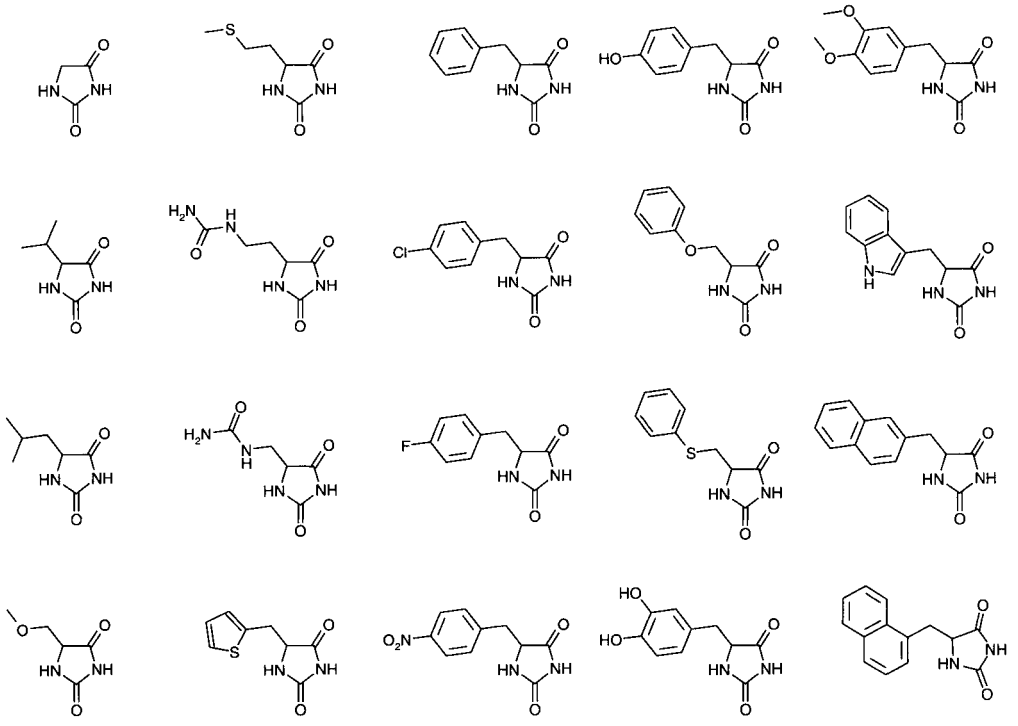


Figure 12.4-16. Substrates accepted by the L-hydantoinases of *Arthrobacter* sp.[7, 51–53] and *Flavobacterium* sp.[46].

Yokozeki et al.[51–53] and Syldatk et al.[7, 125] as well as in *Flavobacterium* sp. by Nishida et al.[46]. These so called "L-5-arylalkylhydantoinases" have comparable substrate specificities and are especially active towards the hydrolysis of hydantoin derivatives with aromatic substituents, as can be seen from Fig. 12.4-16. They could only be induced by D,L-5-indolylmethylhydantoin or the corresponding *N*-3-methyl derivative of a variety of hydantoins and natural cyclic amides[7, 53, 124, 125].

The L-hydantoinase from *Flavobacterium* sp. was reported to be L-selective. Its optimal pH of 9.7 is remarkably high and its optimal temperature is 40 °C[46].

The enzyme from *Arthrobacter aurescens* DSM 3745, which has been crystallized and used for initial X-ray analytical studies[126], was described in detail by May et al.[127–130]. The active enzyme is a tetramer consisting of four identical subunits, each with a molecular mass of 49 670 Da[127], containing 10 mol of zinc per mol of active enzyme, which could be detected by atomic absorption spectrometry and inductive coupled plasma-atomic emission spectrometry[128]. By kinetic studies of metal/chelator enzyme inactivation and by identification of specific metal binding ligands, the role of the zinc atoms was found to be in the catalytic activity as well as in the stabilization of the quaternary structure of the hydantoinase[130]. A reaction mechanism was proposed by Syldatk et al.[13] along the lines published for ureases by Jabri et al.[131] and is shown in Fig. 12.4-17.

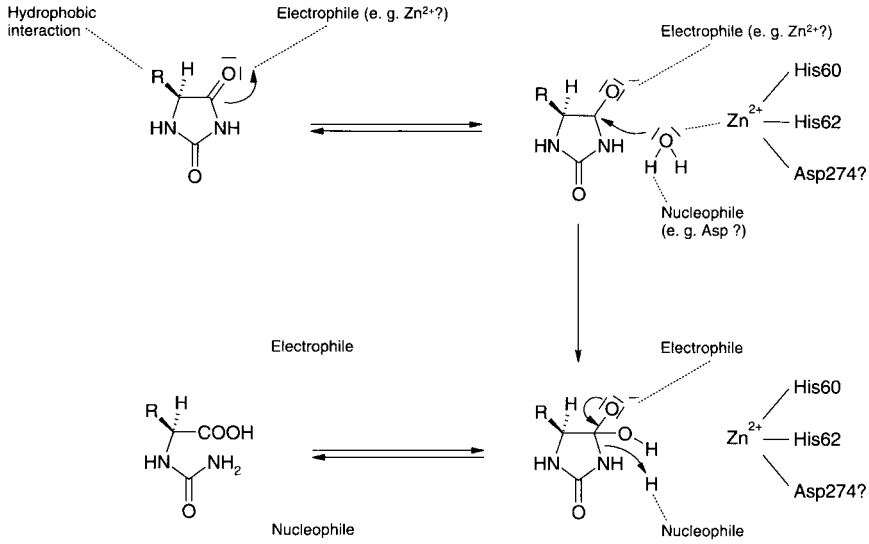


Figure 12.4-17. Proposed reaction mechanism catalyzed by the hydantoinase: after binding of the substrate, an electrophilic residue (or zinc) stabilizes the negative charge of the carbonyl oxygen. Zinc-bound water is activated and performs a nucleophilic attack on the C4 carbon atom, generating a tetrahedral intermediate. The tetrahedral intermediate undergoes ring-opening, assisted by protonation of the ring nitrogen[13]. The proposed residues are derived from a conserved sequence pattern and their respective function in urease[131].

The enantioselectivity of the enzyme was shown to be strongly dependent on the substrate used[127]: while the enzyme is strictly L-selective for the cleavage of D,L-5-IMH, it appears to be D-selective for the hydrolysis of D,L-5-MTEH[127]. As part of these investigations, a method based on enzyme activity stain was developed for the detection of hydantoinases with respect to their enantioselectivity[129]. The isolated enzyme from *Arthrobacter aurescens* DSM 3745 was recently used for the chemoenzymatic production of optically pure D-(trimethylsilyl)alanine[119]. A good stability for the continuous conversion of D,L-5-indolylmethylhydantoin to *N*-carbamoyl-L-tryptophan of $t_{1/2} > 720$ h was first achieved after immobilization of the enzyme by covalent binding to Eupergit C[132]. Further optimization of the immobilization of hydantoin cleaving enzymes has been subsequently carried out[133, 134].

12.4.5
L-*N*-Carbamoylases – Substrate Specificity and Properties

In contrast to the D-route, *N*-carbamoyl-L-amino acid amidohydrolases (L-*N*-carbamoylases) were identified in all L-hydantoinase containing microorganisms discussed in Section 12.4.4 (see above). In this section, L-*N*-carbamoylases from twelve bacterial strains will be discussed with respect to their enzymatic properties and substrate specificities (Table 12.4-5).

The biological function of these enzymes is still unknown, with the exception of

the Mn^{2+}/Fe^{2+}-dependent L-selective β-ureidosuccinase from *Clostridium oroticum* (= *Zymobacterium oroticum*) found by Lieberman and Kornberg in 1955 and postulated to play a role in the degradation of orotic acid[26]. This hydrolase works best at pH 7.8 to 8.5 and its biological function is postulated to be the conversion of *N*-carbamoylaspartic acid into L-aspartic acid. It has not been investigated from the biotechnological aspects as yet.

The twelve L-*N*-carbamoylases derive from seven genera of bacteria: *Alcaligenes* (1), *Arthrobacter* (1), *Bacillus* (4), *Blastobacter* (1), *Clostridium* (1), *Flavobacterium* (1), and *Pseudomonas* (3). Only four of the twelve enzymes have been purified to homogeneity, making a comparison of enzymatic properties difficult. Two of the *Bacillus* strains have been reported to be thermophilic and the enzymes enriched from these strains have been found to possess optimal temperatures approximately 10 to 20 °C higher than most of the other enzymes (Table 12.4-5). The pH-optima of all L-*N*-carbamoylases are between pH 7.5 and 8.5. Whereas hydantoinases are not always strictly L-specific[127], a strictly L-specific carbamoylase, responsible for the optical purity of the amino acid produced with resting cells, has been identified in each strain.

The L-*N*-carbamoylases from *Alcaligenes, Arthrobacter, Bacillus brevis* AJ-12299, *Bacillus stearothermophilus* NS 1122A and the *Pseudomonas putida* IFO 12996 and *Pseudomonas* sp. NS 671 enzymes have been reported to be (hyper-) activated by one or several of the following heavy metal ions: Mn^{2+}, Co^{2+}, Fe^{2+} Ni^{2+}.

In addition to *N*-carbamoylamino acids some enzymes are able to hydrolyze *N*-formyl- or *N*-acetylamino acids[36, 135–137]. As with the hydantoinases, *N*-carbamoylases accept *N*-protected amino acids of unnatural origin. The enzymes of the different genera differ significantly in their substrate specificities. Aliphatic *N*-carbamoylamino acids are preferentially hydrolyzed by the enzymes from the genera *Alcaligenes, Bacillus,* and *Pseudomonas*. Only the *N*-carbamoylase from *Pseudomonas* strain NS 671[138] accepts aromatic amino acids as well as aliphatic ones. Aromatic L-*N*-carbamoylamino acids are preferentially hydrolyzed by the enzymes from the genera *Arthrobacter* and *Flavobacterium*. The substrates hydrolyzed by these enzymes are shown in Fig. 12.4-18. Interestingly, the L-*N*-carbamoylase from *Pseudomonas putida* IFO 12996 accepts *N*-carbamoyl-β-alanine[36] as a substrate, which is an intermediate of the dihydropyrimidine metabolism (see Fig. 12.4-7). In contrast, β-ureidopropionate is not at all converted by the enzymes from *Alcaligenes, Arthrobacter, Bacillus,* and *Pseudomonas* sp. NS 671 and is converted by *Flavobacterium* only, with a very low relative activity.

As has been shown by HPLC, whole cells of *Alcaligenes xylosoxidans* were able to distinguish not only the configuration of the α- but also that of the β-carbon of *N*-carbamoyl-β-methylphenylalanine: from the mixture of the four diastereoisomers only *threo*-L-β-methylphenylalanine was produced[120, 139].

The enzymes from *Arthrobacter, Bacillus stearothermophilus* NCIB 8224 and NS 1122A, and *Pseudomonas* sp. NS 671 have been cloned and expressed in *E. coli*. The enzymes from *Bacillus* and *Pseudomonas* share approximately 38% sequence identity with the *Arthrobacter* enzyme whereas the 20 amino acids known from the *N*-termini of the enzymes from *Alcaligenes* and *Pseudomonas putida* IFO 12996 are

Table 12.4-5. Comparison of L-specific carbamoylases (modified from reference[136]).

Micro-organism	*Alcaligenes xylosoxidans*	*Arthrobacter aurescens* DSM 3747	*Bacillus brevis* AJ-12299, Mutant No.102	*Bacillus brevis* ATCC 8185	*Bacillus stearothermophilus* NCIB 8224	*Bacillus stearothermophilus* NS 1122A	*Blastobacter* sp. A17p-4	*Clostridium oroticum*	*Flavobacterium* sp. AJ-3912	*Pseudomonas putida* IFO 12996	*Pseudomonas* sp. AJ-11220	*Pseudomonas* sp. NS671
Reference	[135]	[136]	[50]	[28]	[137]	[153]	[112]	[26]	[53]	[36]	[35]	[121]
Purification status	homogeneous	homogeneous	partial	whole cells	crude extract	partial[122]	crude extract[118]	whole cells	partial	homogeneous	crude extract	homogeneous (recombinant enzyme)[138]
• MW SDS (Da)	65 000	44 000 Da (calc. 43 993)			44 000 (calc. 44 120)	44.000				45 000		45 000
• MW native (Da)	134 000 Da (2 subunits)	93 000 (2 subunits)								95 000 (2 subunits)		109 000 (2 subunits)
Optimal Temperature (°C)	35	50	50		60	60			40	60		40
Optimal pH (°C)	8.0–8.3	8.5	7.5									
Cloning and Expression		rec. in *E. coli*			rec. in *E. coli*	rec. in *E. coli*						rec. in *E. coli*
Sequence-identity with *Arthrobacter* L-*N*-carbamoylase (%)	**no**				**38**	**38**				**no**		**37**

Table 12.4-5. (cont.).

Micro-organism	*Alcaligenes xylosoxidans*	*Arthrobacter aurescens* DSM 3747	*Bacillus brevis* AJ-12299, Mutant No.102	*Bacillus brevis* ATCC 8185	*Bacillus stearothermophilus* NCIB 8224	*Bacillus stearothermophilus* NS 1122A	*Blastobacter* sp. A17p-4	*Clostridium oroticum*	*Flavobacterium* sp. AJ-3912	*Pseudomonas putida* IFO 12996	*Pseudomonas* sp. AJ-11220	*Pseudomonas* sp. NS671
References	[135]	[136]	[50]	[28]	[137]	[122]	[112]	[26]	[53]	[36]	[35]	[121]
Substrates accepted[a]	b	b							b	b	b	b
	aliphatic C-α-AS: C-L-Ala (100) C-Gly (75) C-L-Val (28) C-L-Leu (9) C-L-Met (12) C-L-Ile (5) C-D,L-2-aminohexanoic acid (24) C-D,L-Ser (19) C-D,L-Thr (9) C-L-Asn (64)	aliphatic C-α-AS: C-L-Met (17)	aliphatic C-α-AS: C-L-Val (100), C-L-Leu (102), C-L-Ile (84), C-L-Met (73), C-L-Ala (48)		aliphatic C-α-AS: C-L-Met (100) C-D,L-Ala (183) C-L-Glu (112) C-Gly (77) C-L-Leu (28)	aliphatic C-α-AS[c]: C-L-Met (97) C-Gly (71) C-DL-Ala (100) C-DL-Val (100) C-L-Leu (94) C-L-Ile (55) C-DL-Ser (86) C-DL-Thr (94) C-L-Glu (56) C-L-Asn (52)			aliphatic C-α-AS: C-L-Met (24) C-D,L-O-Me-Ser (13) C-L-Ser (5) C-Gly (5) C-L-Leu (3) C-L-Ile (2) C-L-Val (2) C-L-Gln (1) C-L-Asn (1) C-L-Ala (0.5)	aliphatic C-α-AS: C-Gly (16) C-L-Ala (118) C-L-Ser (34) C-DL-α-aminobutyrate (31) C-2-aminovalerate (9) C-D,L-Thr (1) C-D,L-Asp (0.1) C-L-Glu (0.3) C-L-Asn (1.6)	aliphatic C-α-AS: C-L-Val (100) C-L-Met (47) C-L-Ala (44) C-L-Leu (98) C-L-Glu (3) C-L-Asn (2)	aliphatic C-α-AS: C-L-Met (100) C-D,L-Ala (102) C-D,L-Val (106) C-L-Leu (118) C-L-Ile (97)
	aromatic C-α-AS: C-L-Phe (5) Other: formyl-D,L-Ala (13) formyl-D,L-Leu (5) formyl-D,L-Met (5) Acetyl-L-Phe (0.7) Acetyl-D,L-2-aminohexanoic acid (0.06)	aromatic C-α-AS: C-L-Trp (100) C-L-Thienylala (316) C-L-Phe (98) C-L-Tyr (127) other: Formyl-D,L-Trp (98)	aromatic C-α-AS: C-L-Phe (86), C-L-Tyr (45)		aromatic C-α-AS: C-D,L-Phe (< 0.1) C-L-Trp (< 0.1) C-L-Tyr (< 0.1) other: Acetyl-Met (38) Acetyl-Glu (7)	aromatic C-α-AS[c]: C-D,L-Phe (25) C-L-Trp (trace) C-L-Tyr (3)			aromatic C-α-AS: C-D,L-3,4-methylenedioxy-Phe (100) C-L-Phe (82 C-L-Tyr (59) C-L-Trp (55) C-D,L-3,4-dimethoxy-Phe (24) C-D,L-O-benzylserine (15) other: β-ureidopropionate (3)	other: β-ureidopropionate (100) γ-ureidobutyrate (290) β-Ureidoisobutyrate (43) formyl-D,L-Ala (75) Acetyl-D,L-Ala (6)	aromatic C-α-AS: C-L-Phe (10) C-L-Tyr (9)	aromatic C-α-AS: C-D,L-Phe (94) C-L-Tyr (60)

a relative activities in [%] are given in brackets () except[c]. b additional data on substrates *not* hydrolyzed are given in the cited literature. c isolated yield in [%] after 24 h in brackets ().

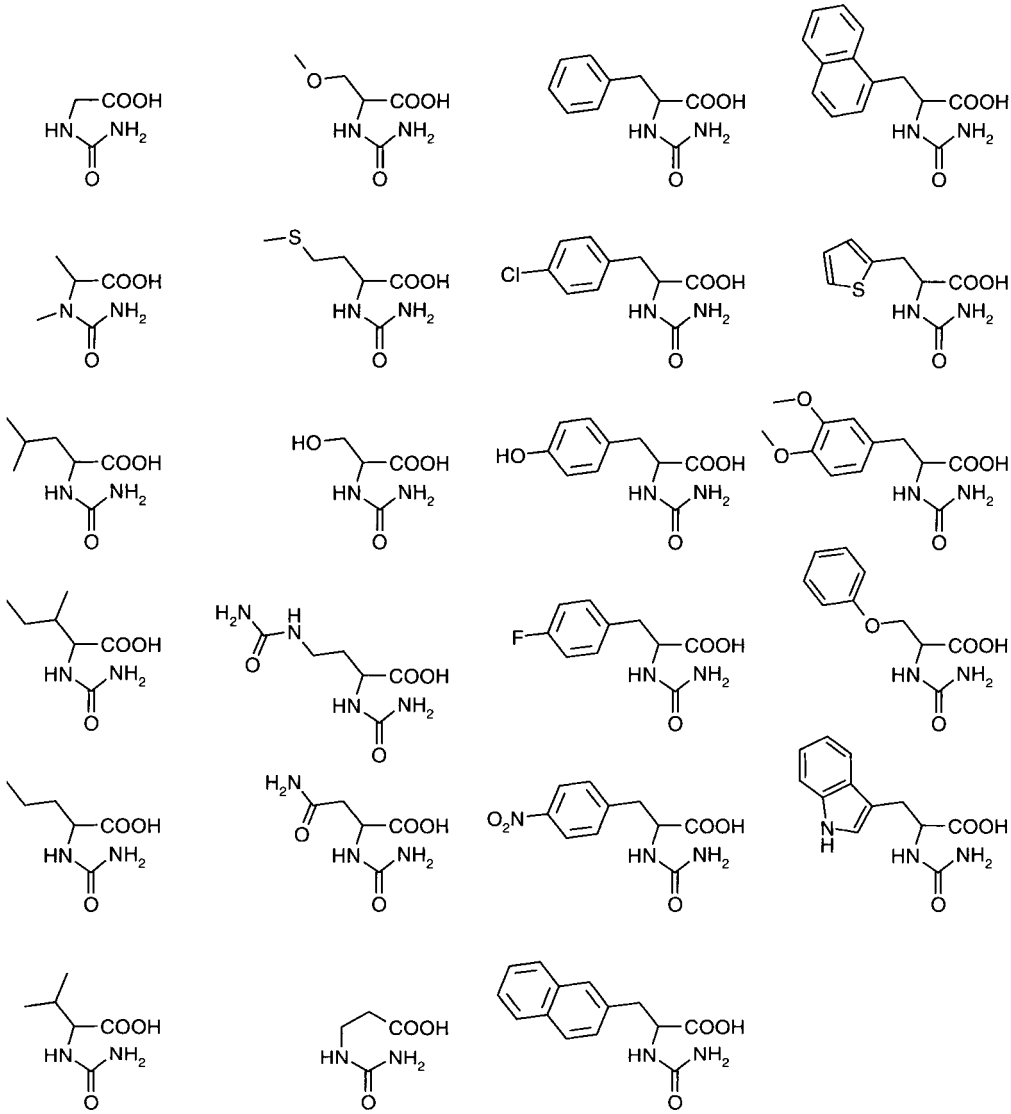


Figure 12.4-18. Substrates accepted by the L-*N*-carbamoylases of *Arthrobacter* sp.[7] and *Flavobacterium* sp.[46, 51–53].

completely different. In contrast to the D-*N*-carbamoylases (see Sect. 12.4.3), the L-*N*-carbamoylase of *Arthrobacter* sp. DSM 3747 is induced by *N*-3-methylated D,L-5-indolylmethylhydantoin, which cannot be hydrolyzed by the cells[7].

Resting cell L-hydantoinase processes were first developed for the industrial production of L-tryptophan by the companies Ajinomoto and Tanabe[46, 51–53]. In 1992 the Rüttgers company tried to enter the amino acid marked with a resting cell

process for the production of unnatural aromatic L-amino acids using *Arthrobacter* sp. DSM 3745 or DSM 3747, which both contain an L-hydantoinase, hydantoin racemase and L-*N*-carbamoylase. However, the productivities obtained (see Fig. 12.4-19 and for details reference[57]) seemed to be too low to fulfill economic requirements.

In recent years, new developments have been published, which could overcome these problems:

1. the L-*N*-carbamoylase from *Arthrobacter aurescens* DSM 3745 and 3747 could be produced as recombinant enzymes in high cell density culture in *Escherichia coli* using an expression system based on the *Escherichia coli rha-BAD*-promoter[140],
2. purification of the recombinant L-*N*-carbamoylases could be optimized by expression of enzymes carrying different tags, making the purification protocols much easier[141] and,
3. the hydantoin-cleaving enzymes from *Arthrobacter aurescens* DSM 3747 could be stabilized significantly by immobilization[133, 134].

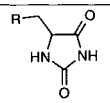
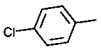
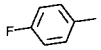
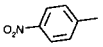
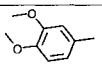
	**Reaction rate (%) after 1 h**	**Molar conversion (%) after ≥ 6 h**	**L-amino acid**
	100	> 90	tryptophan
	140 – 160	> 90	phenylalanine
	20 – 40	> 70	*O*-benzylserine
	150 – 200	> 80	*p*-chloro-phenylalanine
	170 – 200	> 80	*p*-fluoro-phenylalanine
	50 – 70	> 80	*p*-nitro-phenylalanine
	15 – 20	> 70	1'-naphthylalanine
	25 – 30	> 70	2'-naphthylalanine
	25 – 30	> 80	3,4-dimethoxy-phenylalanine
	170 – 200	> 80	2'-thienylalanine

Figure 12.4-19. Industrial production of unnatural aromatic L-amino acids[57].

All these developments, together with the directed evolution of the hydantoinase towards a more L-selective enzyme with higher activity[142] will possibly lead to an economically viable production process in future.

Additionally, an *Escherichia coli* whole cell biocatalyst has been constructed containing the genes of hydantoinase, hydantoin racemase and L-*N*-carbamoylase from *Arthrobacter aurescens* in optimal proportions, so that during the reaction no L-*N*-carbamoyl amino acid occurs as an intermediate product any longer[143].

12.4.6
Hydantoin Racemases

During enzymatic hydrolysis of 5-monosubstituted hydantoin derivatives in some cases the remaining, non-hydrolyzed enantiomer is racemizing chemically under alkaline reaction conditions. The velocity of this chemical racemization is strongly dependent on electronic factors of the substituent in the 5-position (see Table 12.4-6). High velocities of racemization are observed particularly for 5-phenyl- and 5-*p*-OH-phenylhydantoin.

From reports in the early literature resting cell bioconversions of hydantoin derivatives, which do not racemize with high velocities, indicated an enzymatic racemization and the presence of a hydantoin racemase. In addition, the chemical and the enzymatic racemization proceed via the keto-enol tautomerism, which is shown in Fig. 12.4-20. Stabilizing effects on the enolate structure such as electronegative substituents are responsible for the velocity of the racemization[2, 7]. Increased racemization rates can be also seen at more alkaline pH-values and with increased temperatures[7].

The first hydantoin racemase acting on a cyclic amide substrate reported in the literature was the allantoin racemase (E.C. 5.1.99.3) (Fig. 12.4-4). This enzyme enables several bacteria to use both allantoin enantiomers as substrates[20–22]. Racemic mixtures of allantoin, e.g. from plant materials, can be completely metabo-

Table 12.4-6. Racemization rate constants k_{rac} and corresponding half-live times $t_{1/2,rac}$ for various hydantoins at pH 8.5 and 40 °C. Values were calculated from first order rate law: $\ln([a]/[a]_0) = -k_{rac} \cdot t$; $t_{½,rac} = \ln 2/k_{rac}$.

5-Substituted hydantoin	Corresponding D-amino acid	k_{rac} (h^{-1})	$t_{½,rac}$ (h)
Substituent:			
Phenyl	D-Phg	2.59	0.27
Hydroxymethyl	D-Ser	0.43	1.60
Benzyl	D-Phe	0.14	5.00
Methylthioethyl	D-Met	0.12	5.82
1'-Hydroxyethyl	D-allo-Thr	0.11	6.41
3'-Ureidopropyl	D-Cit	0.049	14.26
1'-Methylethyl	D-allo-Ile	0.044	15.84
Imidazolylmethyl	D-His	0.043	16.09
Isobutyl	D-Leu	0.032	21.42
Methyl	D-Ala	0.020	33.98
Isopropyl	D-Val	0.012	55.90

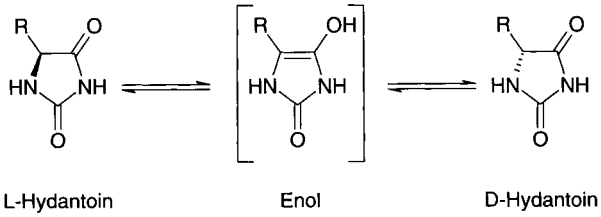
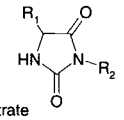


Figure 12.4-20. Keto-enol-tautomerism of 5-monosubstituted hydantoin derivatives.

lized by various bacteria using a sequence of the L-specific allantoinase and allantoin racemase (see Sect. 12.4.1). Although the natural function of this allantoin racemase is not clear, because allantoin racemizes with high velocities under physiological conditions.

The fast and total conversion of L-5-isopropylhydantoin to D-valine by resting microbial cells led Battilotti et al.[30] to the suggestion that a hydantoin racemase might be responsible for the racemization of the L-enantiomer. The first hydantoin racemase to be described in detail was a 5-arylalkylhydantoin racemase, which was isolated and purified from *Arthrobacter* sp. DSM 3747[55, 144, 145]. Its substrate specificity is shown in Fig. 12.4-21.

As can be seen from Fig. 12.4-21, only some aliphatic and aromatic hydantoin derivatives are accepted by the enzyme out of a variety of substrates. The enzyme was recently cloned and heterologously expressed in *Escherichia coli*[146]. The gene encoding the hydantoin racemase, designated *hyuA*, was identified upstream of an L-*N*-carbamoylase gene in the plasmid pAW16 containing genomic DNA of *Arthro-*



Substrate

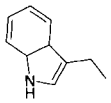
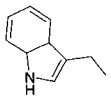
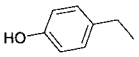
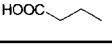
R_1	R_2	Relative activity (%)	R_1	R_2	Relative activity (%)
	- H	100.0		- H	9.8
				- H	20.4
	- CH_3	20.2		- CH_3	0
				- H	0
	- H	76.7		- H	0
	- H	62.7		- H	0
				- H	0

Figure 12.4-21. Substrate specificity of the hydantoin racemase from *Arthrobacter* sp. DSM 3745[55, 144].

bacter aurescens. The matrix assisted laser desorption ionization spectrum (MALDI) of the purified racemase gave a peak at a molecular mass of 25 078.7. This is in good agreement with the calculated value of 25 085 Da for the racemase monomer. On a calibrated column of Superose 12 HR, the relative molecular mass of the native enzyme was estimated to be approximately 170 kDa ± 25, so that the native enzyme is suggested to be either a hexamer, heptamer or octamer. The optimal conditions for racemase activity were pH 8.5 and 55 °C with L-5-benzylhydantoin as the substrate. The enzyme was completely inhibited by $HgCl_2$ and iodoacetamide and stimulated by addition of dithiothreitol, while no effect was seen with EDTA. Kinetic studies revealed substrate inhibition towards the aliphatic substrate L-5-methylthioethylhydantoin. Enzymatic racemization of D-5-indolylmethylenehydantoin in D_2O and NMR analysis showed that the hydrogen at the chiral center of the hydantoin is exchanged for solvent deuterium during the racemization.

Comparative analysis of *hyuA* with various protein databases indicated homology to hydantoin racemases. This hydantoin racemase shared 47.2% identity in amino acid sequence with the hydantoin racemase of *Pseudomonas* sp. NS671 [147] and lower identities to putative hydantoin racemases of *Schizosaccharomyces pombe* (SwissProt accession no. Q09921) and *Saccharomyces cerevisiae* (SwissProt accession no. P32460). The multi-alignment of the enzymes showed that the *N*-terminal region in particular is highly conserved. No significant similarity to the various amino acid racemases or any other racemases deposited was found in the data bases.

The hydantoin racemase from *Pseudomonas* sp. NS 671 is able to racemize both enantiomers of 5-(2-methylthioethyl)hydantoin, 5-isopropylhydantoin, 5-isobutylhydantoin and 5-benzylhydantoin [147]. All together, the presence of hydantoin racemases in resting cells used in industrial processes is of importance for a fast and total conversion of hydantoins which racemize chemically very slowly. In future there might be a combination of hydantoin racemases from L-selective microorganisms with D-hydantoinases and D-*N*-carbamoylases when designing optimal processes leading to D-amino acids. For industrial use, the fast racemization of 5-monosubstituted hydantoin derivatives under mild conditions in the presence of ion exchangers [144, 148] could prove more significant, as this procedure also enables fast and total conversion of D,L-5-monsubstituted hydantoins without enzymatic racemization.

12.4.7 Conclusions

The hydantoinase method has become of significant interest for preparative organic chemistry: total conversion of racemic hydantoins, synthesized by well-established chemical methods to nearly 100% optically pure products is possible using free or immobilized microbial cells or enzymes. Further, it is possible to prepare a wide range of optically pure D- as well as L-amino acids by this method.

Of course there are many factors which influence the competitiveness between enzymatic processes and chemical processes, for example, costs of substrates, costs for production/isolation of enzymes, possible space-time yields and costs for

isolation of the products. These factors are strongly dependent on the desired product and therefore there is no single best process for the production of amino acids. For D-*p*-hydroxyphenylglycine, which is the most important compound produced by the hydantoinase process on an industrial scale (> 1000 tons) at the moment, a first comparison of the feasibility of different methods was given by Tramper and Luyben in the 1980s[149]. However, it has already been shown that the hydantoinase process can be employed for the production of many unnatural amino acids which are components of promising pharmaceuticals[150]. If these pharmaceuticals reach the market, there will be an augmented demand for these amino acids, which could lead to an increased importance of the hydantoinase process in the future.

With the availability of recombinant enzymes, one could expect that the hydantoinase method will also become an important tool in biotransformation of simple precursors to L- and D-amino acids.

Some of the current reports on hydantoinase processes focus on isolation and the recombinant expression of thermostable enzymes[84, 86, 87, 95, 151]. Processes at an elevated temperature would increase the solubility and racemization rate of hydantoins. Therefore, the increased thermostability of these enzymes is very useful, if the specific activities are still high.

Another main advantage of the recombinant expression of the hydantoin cleaving enzymes is to decrease the costs of catalysts, which might contribute to the competitiveness of the hydantoinase processes, which to date do not employ recombinant enzymes. The Kanekafuchi company have published a patent for the production of D-*N*-carbamoyl-amino acid from 5-substituted hydantoin, using a recombinant hydantoinase derived from a strain of *Pseudomonas*, *Agrobacterium* or *Bacillus*[152]. This might indicate that highly active recombinant *Escherichia coli* cells could replace the wild-type cells in the near future. Furthermore, the recombinant expression of hydantoinases (and of course carbamoylases[153, 154]) allows enzyme properties such as stability or stereoselectivity to improve by means of protein design. If an X-ray structure was solved, this could be done by a rational protein design[155] or, lacking knowledge about a structure, by evolutionary protein design[156]. May et al. are already able to improve the stereoselectivity of a L-hydantoinase for the conversion of D,L-5-methylthioethylhydantoin[142], while Kim et al. have shown the possibility of using fusion proteins of D-hydantoinase and D-*N*-carbamoylase for the production of D-amino acids[157, 158].

Future work will show the impact of these methods on the biotechnological application of hydantoinases. Besides the applied research on hydantoinases for the production of amino acids, the natural functions and genetic organization of distinct hydantoinases, related hydantoin racemases and *N*-carbamoylases are still unknown and are of great interest for basic research.

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12.5
Hydrolysis and Formation of Peptides

Hans-Dieter Jakubke

12.5.1
Introduction

Peptides and proteins play a fundamental role in the formation and maintenance of structure and function of living systems. Peptides comprise a variety of biologically active linear and cyclic compounds with diverse functions. The different classes of peptides include, for instance, hormones and other signalling or regulatorry factors, antibiotics, alkaloids, toxins, enzyme inhibitors, and sweeteners. There is permanently great interest in pharmaceutically active peptides and proteins since they have many applications and great potential in medicine, such as in cardiovascular diseases, mental illness, connective tissue diseases, the therapy of cancer, regulation of fertility and growth, and the control of pain. The demand for peptides and proteins is enormous, and rising all the time.

In a peptide chain amino acids are linked together by bonds between the carboxyl group of one and the amino group of another amino acid, known as peptide bonds. This amide or peptide bond has some characteristics of a double bond: it does not rotate freely and is shorter than other C - N bonds. Nature provides a wide range of special enzymes, the proteolytic enzymes or correctly designated as peptidases, which can cleave these bonds in peptide and protein substrates. In contrast, for catalyzing the formation of peptide bonds the number of efficient enzymes is rather low. Peptidases catalyze a single reaction, the hydrolysis of a peptide bond. The ubiquitous distribution among all life forms and their enormous diversity of function makes the peptidases one of the most fascinating families of enzymes. As a result of complete analysis of several genomes it has been shown that about 2 % of all gene products are proteolytic enzymes. In biological and biochemical research proteolytic enzymes play a contrary role: some researchers either love them or other hate them. In the first case, the only good peptidase is a dead one, no longer capable of degrading the desired protein during isolation and purification. Irreversible inhibition of any contaminating proteolytic enzyme is the best way to solve this problem. However, for most purposes proteolytic enzymes are of great importance. Owing to the special physiological functions, some proteolytic enzymes are active in degrading proteins for digestive and nutritional purposes. These enzymes act both extracellulary (e. g. in the intestine of animals) and intracellulary (in the hydrolytic subcellular organelles, preferentially in liver and kidney cells). Other peptidases are responsible for controling processes, e. g. they can act to cause limited proteolysis of peptide and protein substrates. In limited proteolytic processes a single susceptible peptide bond may be cleaved followed by a dramatic change in the biological activity of the products formed. Physiological functions are a result of proteolytic conversion of inactive precursors into biologically active proteins, e. g. in blood coagulation, prohormone or proenzyme activation. Pancreatic peptidases frequently exist as

zymogens, a special inactive proenzyme arrangement that ensures that the pancreas does not digest itself. These enzymes have their function outside cells and will be activated by another peptidase at the place of action. The number of peptidases within the cell are more numerous but much more difficult to investigate in comparison with the extracellular enzymes[1]. A much smaller group are the cell-surface peptidases which are specialized in the hydrolysis of relatively simple peptides rather than proteins. This group of peptidases does not need activation. Usually the biological function is the inactivation of signalling peptides in order to terminate a hormonal or neuropeptide signal but sometimes they activate peptide substrates, e.g. the conversion of angiotensin I to angiotensin II[2, 3].

Contrary to the well-known native function of peptidases the reverse reaction, the peptidase-catalyzed peptide bond formation, can only be successfully carried out by manipulating the reaction conditions, the enzyme or the substrate. Besides enzymatic techniques, classical chemical synthesis in solution, solid-phase synthesis and recombinant techniques belong to the most important methods of peptide synthesis.

The main aim of this chapter is to give an overview of the present importance of proteases in the technology of peptide synthesis.

12.5.2 Hydrolysis of Peptides

12.5.2.1 Peptide-Cleaving Enzymes

12.5.2.1.1 Introduction and Terminology

More than 500 proteolytic enzymes are known and, in a general sense, they all catalyze the same reaction: hydrolysis of peptide bonds. An excellent handbook[4] provides a ready reference to the approximately 500 proteolytic enzymes known up to the end of the 1990s. These enzymes are classified as peptidases or proteases. In the past there has been widespread uncertainty about the exact meaning of the terms *proteases, peptidases* and *proteinases,* as well as *proteolytic enzymes.* There is no doubt that proteolytic enzymes was the most generally understood term in the current usage. However, this is ambiguous since many of the enzymes which are capable of hydrolyzing peptide bonds do not accept proteins as substrates. The Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB) recommends the term ***peptidase*** as the general term for all peptide bond-hydrolyzing enzymes. The E.C. List can be found in its revised version on the World Wide Web (www) at http://www.chem.qmw.ac.uk/iubmb/enzyme/index.html.

The acceptable terms for the major types of peptidases are shown in Fig. 12.5-1. The meanings of the words below are described by the italicized semi-systematic terms. The terms in bold type are preferred, whereas the terms in parentheses have historical precedence and are satisfactory when used in the correct context. Most of the peptidases fall into one of two categories, depending on the positional specificity of the peptide bond cleavage process. An enzyme is said to be an ***endopeptidase*** when

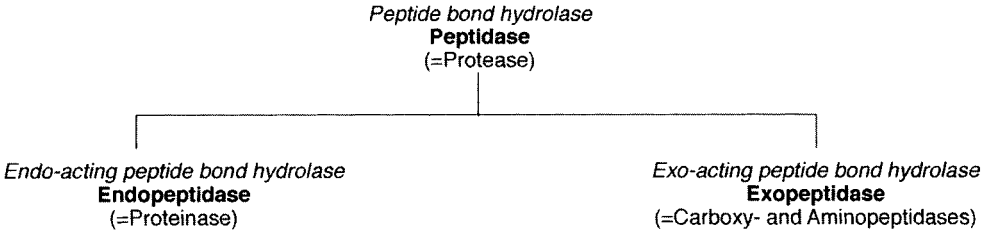

Figure 12.5-1. Proposed terms for the major types of peptidases.

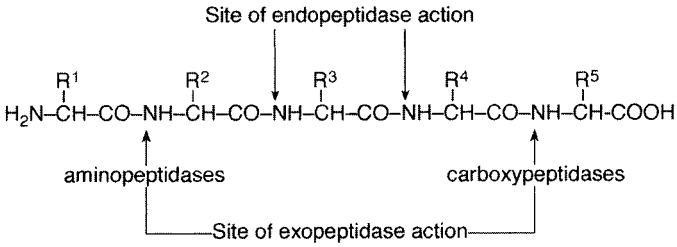

Figure 12.5-2. Scheme of the action of endopeptidases and exopeptidases.

the susceptible peptide bond is an internal one in a peptide or protein. In contrast, an enzyme is termed an ***exopeptidase*** when the susceptible peptide linkage is at the carboxyl terminus or at the amino terminus of the substrate. In the E.C. List there are also terms for subtypes of exopeptidases and endopeptidases. Exopeptidases acting at the free *N*-terminus liberating a single amino acid residue (***aminopeptidases***) or a dipeptide or a tripeptide (***dipeptidyl-peptidases*** and ***tripeptidyl-peptidases***), whereas those acting at the free *C*-terminus liberate a single residue (***carboxypeptidases***) or a dipeptide (***peptidyl-dipeptidases***). Furthermore, other exopeptidases are specific for dipeptides (***dipeptidases***) or remove terminal residues which are substituted, cyclized or linked by isopeptide bonds (***omega peptidases***). Endopeptidases act on bonds in the middle of the peptide chain (see Fig. 12.5-2). The term ***oligopeptidase*** is used to refer to endopeptidases that act optimally on oligopeptide substrates rather than on proteins.

Peptidases differ in the specificities that they display in a hydrolysis reactions. It is somewhat simplistic to designate a peptidase on the basis of a single amino acid residue at the active site. Near the active site of the peptidase is a "pocket" in the surface of the enzyme molecule which is specific for amino acid side chains of the substrate. Owing to different interactions in this region there are great differences in the so-called primary specificity of the peptidases. Trypsin, for example, cleaves only those peptide bonds adjacent to the amino acids lysine or arginine which carry a positive charge and are hydrophilic. In the binding pocket of trypsin a negatively charged aspartic acid unit is at the back, holding the positively charged lysine or arginine side chain in the pocket by electrostatic forces. Despite the fact that this pocket for specific side chains is obviously important for binding, it is not the only binding site. It has been followed from kinetic studies that the binding of substrates (and inhibitors) involved interactions at a number of subsites on either side of the

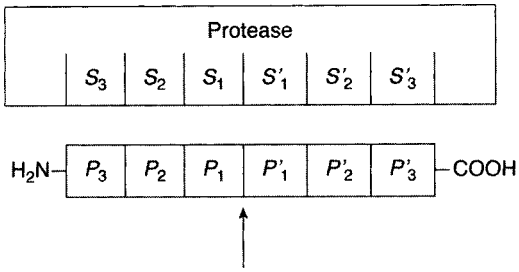

Figure 12.5-3. Simplified representation of the peptidase specificity according to Schechter and Berger[255]. The amino acid residues of the substrate are denoted by P and P', respectively. They interact with the corresponding S and S' subsites of the enzyme active site, respectively. The arrow indicates the site of hydrolytic cleavage.

pair of residues containing the peptide bond to be hydrolyzed. The enzyme and substrate must be fixed at several points, so that the susceptible bond is oriented at the active site in optimal configuration.

In 1967, a system of nomenclature to describe the interaction of peptidases and their substrates was introduced by Schechter and Berger[255]. According to this system the binding site for a peptide substrate in the active site of a peptidase is envisioned as a series of subsites S which interact with the amino acid building blocks P of the peptide or protein substrate (see Fig. 12.5-3). The amino acid residues of the substrate are denoted by P and P', respectively, which interact with the corresponding S and S' subsites within the active site of the peptidase. The sites are numbered from the catalytic site, $S_1 \ldots S_n$ towards the N-terminus of the peptide substrate, and $S_1' \ldots S_n'$ towards the *C*-terminus. In analogy, the residues which they accommodate are numbered $P_1 \ldots P_n$, and $P_1' \ldots P_n'$, respectively. The arrow indicates the site of enzymatic cleavage of the substrate between the residues P_1-P_1'. With the increasing knowledge of the amino acid sequences of peptidases and particularly when the three-dimensional protein structure began to emerge, a functional division of peptidases became possible. Detailed mapping of the active sites has provided a better understanding of the interaction of substrate and peptidase and has permitted both the design and synthesis of highly specific inhibitors as well as a useful prediction of the outcome of the reverse peptidase action in peptide synthesis (see Sect. 12.5.3.3).

The general stoichiometry for the hydrolysis of a peptide bond is shown in Fig. 12.5-4.

Water attacks the electron-deficient carbonyl atom targetting first a tetrahedral adduct, which then eliminates the amine fragment and produces the acid. The process is characterized by transferring the aminoacyl moiety of the peptide to water. In this type of group-transfer reaction the nucleophilic co-substrate is water; 55.5 M water is the most nearly ubiquitous weak nucleophile in degradative enzymatic

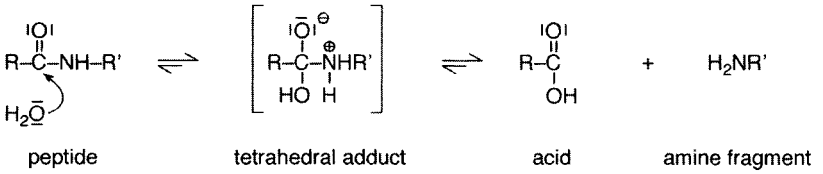

Figure 12.5-4. The general mechanism for the hydrolysis of a peptide bond.

processes in the cell. Under physiological conditions the hydrolysis of peptide bonds will proceed in the absence of peptidases, but only at an exceedingly low rate. The reactants only rarely attain the high internal energy required for the hydrolysis process.

In contrast, enzymes allow the reaction to follow a different pathway from the substrate to the products, and, therefore, reduce the energy barriers. In the course of the reaction new intermediate states of highest energy appear, with energy lower the internal energy barriers, e.g. the high-energy transitions between one intermediate and the following one.

Proteolysis is functionally irreversible, since energy is liberated in the hydrolysis of peptide bonds. From the overall change in energy it follows that the ionized hydrolysis products are thermodynamically more stable.

On the other hand, aminoacyl-group transfer is involved in protein biosynthesis. As a result of the ionized state of amino acids at physiological pH, the attack by the amino group of another amino acid to form a peptide bond would involve formal expulsion of O_2^{2-}. This species is very instable and, therefore, would not proceed to any reasonable extent. In protein biosynthesis the carboxylate must be chemically modified so that an oxygen atom can be eliminated with a low energy activation. The key concept in protein biosynthesis is that the aminoacyl group from an activated intermediate is transferred to the specific nitrogen of the amino group catalyzed by the ribosomal peptidyltransferase. The reaction takes place via the transfer of a peptidyl residue from peptidyl-tRNA in the ribosomal P site to the amino group of the aminoacyl-tRNA in the A site.

Despite many years of intensive research, the nature and the basic mechanism of the ribosomal peptidyltransferase reaction is still largely unknown. Recently, Zhang and Cech[5] demonstrated that an *in vitro*-selected ribozyme can catalyze the same type of peptide bond formation as a ribosome. The ribozyme resembles the ribosome in such a way that a very specific RNA structure is necessary for substrate binding and catalysis, and both amino acids to be coupled are attached to nucleotides. Despite the presence of many different possible peptidyltransferase ribozymes, one of these must be strikingly similar in sequence and secondary structure to the "helical wheel" portion of 23S rRNA implicated in the activity of the ribosomal peptidyltransferase. These results from Cech's group demonstrate that a ribozyme is capable of catalyzing peptide bond formation analogous to the action of the ribosome, providing evidence that RNA itself can make peptides and support the "RNA world" hypothesis in biological evolution.

Since the ribosomal peptidyltransferase activity is not suitable for practical use as a simple C-N ligase and, in addition, the multienzyme complexes involved in bacterial peptide synthesis[6] do not seem to possess a general applicability, only the reverse catalytic potential of peptidases can be considered as valuable supplement to chemical coupling methods (cf. Sect. 12.5.3). In addition, peptidases have been used successfully for enzymatic manipulation of protecting groups in peptide synthesis[7–9].

12.5.2.1.2 Catalytic Mechanism[10, 11]

The overall process of peptide bond scission is identical in all classes of peptidases and differences between the catalytic mechanisms are rather subtle. The attack on the carbonyl group of the peptide bond requires a nucleophilic agent, either oxygen or sulfur, in order to approach the slightly electrophilic carbonyl carbon atom. To remove a proton from the attacking nucleophile, general base catalysis will assist this process. Furthermore, some type of electrophilic action on the carbonyl oxygen increases the polarization of the C - O-bond.

Generally, the four classes of peptidases (serine, cysteine, aspartic and metallopeptidases) differ in the groups that perform nucleophilic attack, general base catalysis, and electrophilic assistance. Also, different groups are involved in the breakdown of the tetrahedral intermediate which is formed in the initial nucleophilic attack, requiring general acid catalysis to promote the departure of the amine fragment. The four types of peptidases are based on the different catalytic mechanisms, which were first recognized by the use of some group-specific inhibitors.

The reactive serine residue in the active site of serine peptidases (but also in other serine hydrolases, such as acetylcholine esterase) react in an irreversible step with organophosphate compounds, e.g. diisopropyl phosphofluoridate (DFP or DipF) resulting in the death of the appropriate enzyme. Owing to the high toxicity of DFP other reagents, e.g. phenylmethylsulphonyl fluoride (PMSF) and 3,4-dichloroisocoumarin (3,4-DCl) have been used in its place. The reactive cysteine residue of cysteine peptidases is susceptible to oxidation and can react with various reagents: iodoacetate, *N*-ethyl-maleimide, heavy metals (for example Hg) and with the highly selective inhibitor *N*-[L-3-*trans*-carboxyoxiran-2-carbonyl-L-leucyl-amido(4-guanidino)butane] (E-64). The highly acidic pH optima led to the first recognition of aspartic peptidases. Later, with pepstatin A from a strain of *Streptomyces*, a specific inhibitor was found. Chelating agents, e.g. EDTA and 1,10-phenanthroline are prone to inhibit metallopeptidases.

Serine Peptidases[12]

These form the most studied class of peptidases. They have a reactive serine residue, e.g. the hydrolysis of a peptide substrate involves an acylenzyme intermediate in which the hydroxyl group of Ser^{195} (from the chymotrypsin numbering system) is acylated by the acyl moiety of the substrate, releasing the amine fragment of the substrate as the first product. The formation of the acylenzyme is the slow step in peptide bond hydrolysis, but the acylenzyme often accumulates in the hydrolysis of ester substrates. The acylenzyme thus formed will be the same for a series of substrates which differ in their leaving group.

The catalytic mechanism of serine peptidases will be given in terms of chymotrypsin (Fig. 12.5-5). After chymotrypsin has bound the substrate to form the Michaelis complex, the attack of Ser^{195} on the peptide bond of the substrate forms a high energy tetrahedral intermediate. At the same time the proton of the serine hydroxyl group is transferred to the nearby His^{57}, the serine hydroxy group forms a covalent bond with the carbonyl atom of the peptide bond to be cleaved. The liberated proton is taken by the imidazole ring of His^{57} thereby forming an imidazolium ion

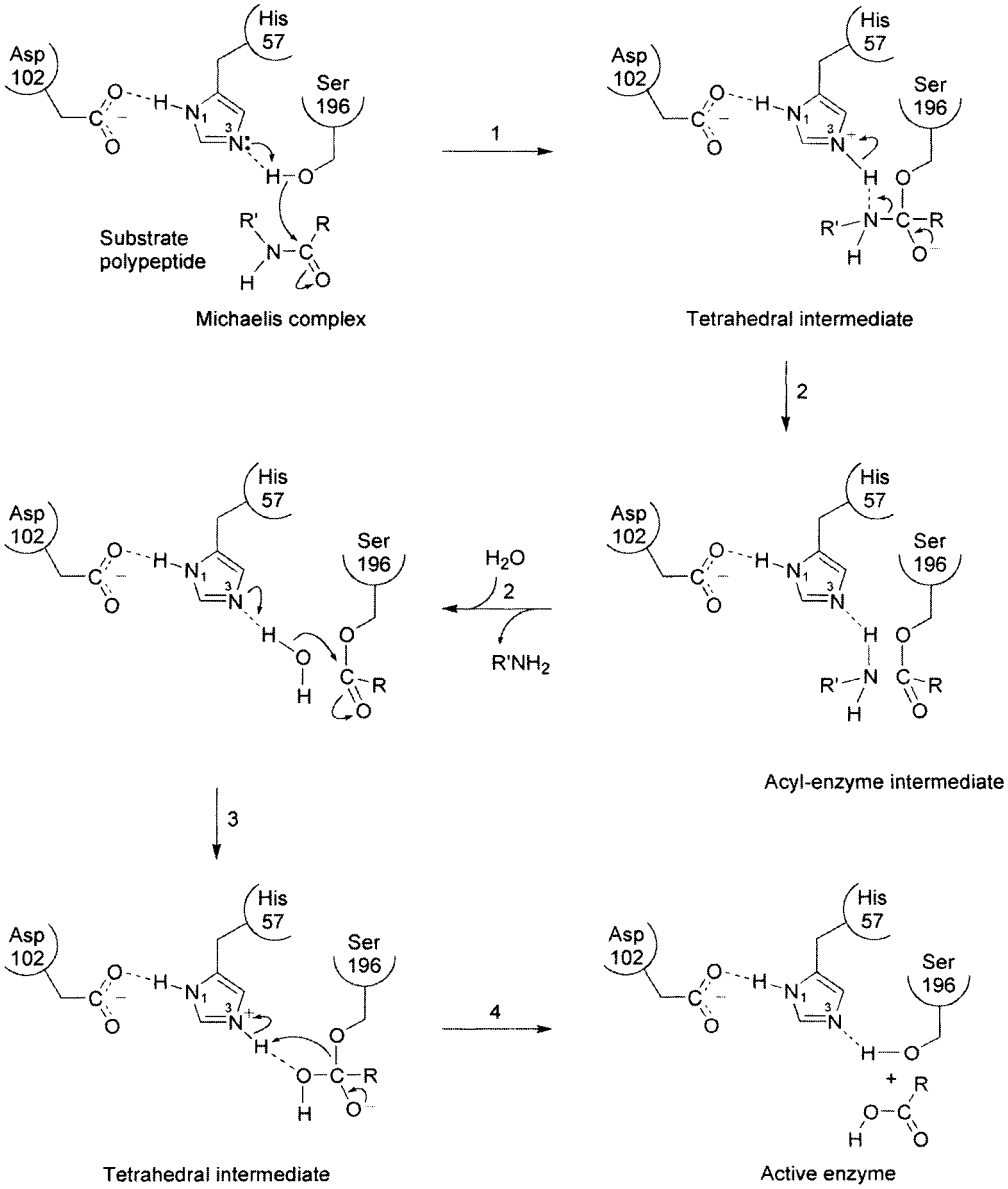

Figure 12.5-5. Scheme of the catalytic mechanism of serine proteases (chymotrypsin numbering).

(general base catalysis). This process is supported by the polarizing effect of the unsolvated carboxylate ion of Asp^{102} which is hydrogen bonded to His^{57} in the sense of electrostatic catalysis. Mutagenic replacement of Asp^{104} by Asn in trypsin, for example, did not changed the K_M substantially at neutral pH. On the other hand, k_{cat}

was reduced to < 0.05% of its wild-type value. Furthermore, neutron diffraction studies have shown that Asp^{104} remains as a carboxylate ion rather than a proton being abstracted, as from the imidazolium ion of His^{57} to form an uncharged carboxylic moiety. The active site of serine peptidases is complementary in structure to the transition state of the reaction, a structure which is very close to the tetrahedral adduct of Ser^{195} and the carbonyl carbon of the peptide substrate. Indeed, transition state binding catalysis provides the catalytic power of the appropriate serine peptidase.

In the course of the formation of the tetrahedral intermediate a conformational distortion causes the carbonyl oxygen of the scissile peptide bond to move deeper into the active site to occupy the oxyanion hole. The resulting oxyanion is hydrogen-bonded to the backbone of NH groups of Gly^{193} and Ser^{195}, whereas the NH group of the peptide bond preceding the scissile bond forms a hydrogen bond to the backbone carbonyl of Gly^{193}. The decomposition of the tetrahedral intermediate forming the acylenzyme intermediate and the amine product occurs under the driving force of proton donation from the N3-atom of His^{57} through general acid catalysis. The N-terminal part of the cleaved peptide chain (amine product) will be released in the next step and replaced by a water molecule forming a second tetrahedral intermediate. The latter decomposes to the reaction's carboxyl product (*C*-terminal portion of the cleaved peptide chain) and the active enzyme. Generally, all the serine peptidases employ the same catalytic three amino acid units to hydrolyze peptide bonds. The diversity of serine peptidases results entirely from the way they accommodate their specific substrates.

Cysteine Peptidases[12]

Other terms for cysteine peptidases are cysteine-type peptidases, thiol peptidases or sulfhydryl peptidases. They are peptidases in which the attacking nucleophile is the sulfhydryl group of a cysteine residue (Cys^{25} in the papain numbering system). The mechanism of catalysis is similar to that of serine peptidases because a covalent intermediate is formed. Beside the cysteine nucleophile a proton donor/general base is required, which in the majority of cysteine peptidases is a His residue (His^{159}). Despite the fact that in some families of cysteine peptidases a third amino acid residue is required to orientate the imidazolium ring of the histidine moiety in the course of the catalytic process, in general, only a catalytic dyad is necessary.

The archetypal cysteine peptidase is *papain* which was isolated from the latex of the tropical papaya fruit (*Carica papaya*)[13, 14]. It is a single protein of 212 amino acid residues containing three disulfide bonds and the three-dimensional structure is known with 1.65 Å resolution[15]. The catalytic amino acid residues have been identified as Cys^{25}, His^{159} and Asn^{175}, whereas Gln^{19} helps to stabilize the oxyanion hole. A second group of cysteine peptidases which is very diverse in sequence is the *"papain-like" endopeptidases of RNA viruses* containing only the catalytic dyad Cys/His without any additional residues being involved in the catalytic mechanism. The same is true for *caspases*, a group of ten cytosolic endopeptidases with strict specificity for cleavage of aspartyl bonds. *Clostripain* from the anaerobic bacterium *Clostridium histolyticum* is a heterodimeric protein of 526 amino acid residues. The

heavy chain ($M_r \sim 43000$ Da) and the light chain ($M_r \sim 15398$ Da) are held together by strong noncovalent forces rather than by disulfide bridges. Cys^{41} of the heavy chain was identified as the catalytic residue of the active site. This peptidase is well known for the selective cleavage of arginyl bonds, whereas lysyl bonds are hydrolyzed at a lower rate. The catalytic mechanism of the *adenovirus endopeptidase* is similar to that of papain, the difference being that four amino acids His, Glu (or Asp) Gln and Cys are involved in it.

Last but not least, the *caspases* with a strict specificity for cleavage of aspartyl bonds should be mentioned as the last family of cysteine peptidases. Members of this family transmit the events leading to apoptosis of animal cells.

Aspartic Peptidases

The aspartic peptidases comprise peptidases which catalyze the hydrolysis of peptide bonds without the use of nucleophilic attack by a functional group of the enzyme. The nucleophile attacking the scissile peptide bond in this case is an activated water molecule and no covalent intermediate will be formed between the enzyme and a fragment of the substrate. The name of this group of peptidases is based on the catalytic domain which consists of two aspartic acid side chains (Asp^{32} and Asp^{215} of the porcine pepsin numbering system) activating the water molecule directly. These two side chain carboxyl groups are close enough to share a hydrogen bond between two of their oxygens holding the water in place. However, there are not two Asp residues in the catalytic dyad in all members of aspartic peptidases. An endopeptidase from *nodavirus* has an Asp and an Asn as catalytic residues, and in a related *tetravirus* endopeptidase the Asp residue is replaced by Glu. It is interesting to note that all the enzymes so far described are endopeptidases.

Metallopeptidases

As with the aspartic peptidases, metallopeptidases do not form covalent intermediates and the nucleophilic attack on the peptide bond to be cleaved is mediated by a water molecule. The latter is activated by a divalent metal cation, usually Zn^{2+} but sometimes also Co^{2+} or Mg^{2+}. In order to assist in attack of a water molecule the metal ion provides a strong electrophilic "pull". The metallopeptidase has a water molecule coordinated to the fourth tetrahedral site. Beside the metal ion the other ligands are two histidine building blocks and a glutaminic acid residue in thermolysin and carboxpeptidase A. The enzymes of this family can be divided in two groups depending on the number of metal ions necessary for catalysis. In many cases only one zinc ion is required, but often two metal ions act cocatalytically. All the enzymes which contain cobalt or manganese require two metal ions, but zinc-dependent enzymes are also known in which two zinc ions act in a cocatalytic manner. Enzymes known to date containing cocatalytical metal ions are exopeptidases, whereas those with one catalytic metal ion belong to exopeptidases or endopeptidases. His, Glu, Asp or Lys are known metal ligands in metallopeptidases. Together with the metal ligand very often a Glu residue is engaged in the catalytic process. In the leucyl aminopeptidase Lys or Arg fulfill this function.

12.5.2.1.3 E.C. Classification

As shown above, based on the chemical groups that are responsible for their catalytic activity, peptidases have been classified into four distinct groups. Recommended by the International Union of Biochemistry and Molecular Biology (1992)[16] all hydrolases are designated as E.C. 3., and the peptidases as E.C. 3.4. defining the main classes of peptidases by a third numeral (11 to 24) as indicated in Table 12.5-1. The sub-subclasses are not further divided. The enzymes are listed in arbitrary order within each of them. Unfortunately, the molecular structures and evolutionary relationships are not taken into account in the E.C. classification. In this E.C. list the exopeptidases are mainly classified based of their action. Generally, only peptides with an unblocked terminus are attacked. The only exception are so-called *omega peptidases* which comprise a very small number that are capable of releasing certain modified terminal residues. To this group belong, for example, acylaminoacyl peptidases which release acetyl or formyl moieties from the *N*-terminus, and the pyroglutamyl peptidase, capable of releasing the cyclic residue. An isopeptide bond will be cleavaged by the β-aspartyl peptidase. Other omega peptidases are directed to the substituted *C*-terminus, e.g. the peptidyl glycinamidase releasing a *C*-terminal glycine amide, and the γ-glutamyl carboxypeptidase which splits a *C*-terminal glutamic acid linked by an isopeptide bond.

12.5.2.1.4 Peptidase Families and Clans

Starting with the earlier work of Rawlings and Barret[17] and improved in the handbook[4] another level of sophistification to the classification of peptidases has been developed. Evolutionary considerations can be taken into account due to the relative ease by which cDNA-derived sequences can now be obtained. According to this principle of classification a *family* of peptidases is defined as a group in which

Table 12.5-1. Principles of peptidase classification according to the Enzyme Commission (E.C.) of the International Union of Biochemistry and Molecular Biology[16].

E.C. Number	Type of peptidase	Type of cleavage
Exopeptidases		
3.4.11.-	Aminopeptidase	N-terminal residue
3.4.14.-	Dipeptidase	Dipeptides only
3.4.14.-	Dipeptidyl peptidase	*N*-terminal dipeptide
	Tripeptidyl peptidase	*N*-terminal tripeptide
3.4.15.-	Peptidyl dipeptidase	*C*-terminal dipeptide
3.4.16.-	Carboxypeptidase (serine)	*C*-terminal residue
3.4.17.-	Carboxypeptidase (metallo)	*C*-terminal residue
3.4.18.-	Carboxypeptidase(cysteine)	*C*-terminal residue
3.4.19.-	Omega peptidase	Terminal modified residue
Endopeptidases		
3.4.21.-	Serine endopeptidase	
3.4.22.-	Cysteine endopeptidase	
3.4.23.-	Aspartic endopeptidase	
3.4.24.-	Metalloendopeptidase	
3.4.99.-	Endopeptidase with unknown mechanism	

every member indicates a statistically significant relationship in the amino acid sequence to at least one other member of the family in the part of molecule which is responsible for peptidase activity. Applying strict statistical criteria implies confidence that any two peptidases that are placed in the same family have evolved from a common ancestor and thus are homologous according to the definition of Reeck et al.[18]. Each peptidase family is named with a letter that denotes the catalytic type (S, T, C, A, M or U, for serine, threonine, cysteine, aspartic acid, metallo- or unknown), followed by an arbitrarily assigned number (see Table 12.5-2). The term *clan* is used for defining a group of families the members of which have evolved from a single ancestral protein, but have diverged so far that their relationship can no longer proved by comparison of the primary structures. Clan-level relationships between families can at best be made evident by similarities in three-dimensional structures. The name of the clan is formed from the letter for the catalytic type (in analogy to families) followed by an arbitrary second capital letter.

About 40 families of serine- and threonine-type peptidases can be distinguished on the basis of sequence comparison. However, only a few known families of threonine-dependent peptidases are included therein. By comparing the tertiary structures and the order of the catalytic residues in the sequence most of these families can be grouped into seven clans (cf. Table 12.5-2).

The serine peptidases and their clans can be used to demonstrate this type of classification in more details. In *clan SA* with the order of the catalytic triad His, Asp, Ser the tertiary structure is characterized by a β sheet-based two-domain structure. Each domain contains a β barrel and between the domains the active site cleft is located. The largest family S1 of trypsin consists of more than 70 sequenced proteins. Well-known members of the family S2 are, e.g. streptogrisin A, glutamyl endopeptidase, and lysyl endopeptidase (*Achromobacter*). Togavirin (S3), IgA1-specific serine-type prolyl endopeptidase (S6), flavivirin (S7), hepatitis C polyprotein peptidase (S29), helper component proteinase (S30), pestivirus NS2–3/NS3 serine peptidase and arterivirus serine endopeptidase (S32) complete the families of clan SA. The order of the catalytic triad of *clan SB* is Asp, His, Ser and the tertiary structure contains both β sheets and α helices. This clan contains only the subtilisin family (S8) including peptidases from archaea, bacteria and eukaryotes.

Clan SC contains peptidases with the α/β hydrolase fold bearing the catalytic triad in the order Ser, Asp, His. This clan includes the families (characteristic member in parentheses) S9 (prolyl oligopeptidase), S10 (carboxypeptidase C), S15 (Xaa-Pro dipeptidyl -peptidase), S28 (lysosomal Pro-Xaa carboxypeptidase), S33 (prolyl aminopeptidase), and S37 (*Streptomyces* PS-10 peptidase). The characteristic catalytic dyad Ser, Lys of *clan SE* is represented by the motif Ser-Xaa-Xbb-Lys, and the fold consists of helices and an α+β sandwich. The families of this clan S11 (penicillin-binding protein 5), S12 (*Streptomyces* R61 D-Ala-D-Ala carboxypeptidase), S13 (penicillin-binding protein 4) are involved in the biosynthesis, turnover and lysis of bacterial cell walls.

The catalytic residues in *clan SF* (catalytic dyad Ser, Lys or Ser, His) are more widely spaced in comparison with clan SE. The families of this clan include only endopeptidases from bacteriophages, bacteria, archaea and eukaryotes with the members: S24

Table 12.5-2. Evolutionary classification of peptidases into families and clans based on primary and teriary structure.

Class (E.C. list)	Families	Clans (families)	Catalytic residues
Serine (E.C. 3.4.21.)	S1-S44	SA (S1–3,6,7,29–32,35,43)	His, Asp, Ser
		SB (S8)	Asp, His, Ser
		SC (S9,10,15,28.33,37)	Ser, Asp, His
		SE (S11–13)	Ser, Lys
		SF (S24,26,41,44)	Ser, Lys, (His)
		SH (S21)	His, Ser, His
		TA (S42)	Thr
		SX (14,16,18,19,34,38,39,43)	
Cysteine (E.C. 3.4.22.)	C1-C47	CA (C1,2,10,12,19)	Cys, His, Asp (Asn)
		CB (C3,4,24,30,37,38)	His, Cys
		CC (C6–9,16,21,23,27–29,31–36, 41–43)	Cys, His
		CD (C14)	His, Cys
		CE (C5)	His, Glu(Asp), Gln, Cys
		CX (C11,13,15,22,25,26,39,40)	
Aspartic (E.C. 3.4.23)	A1-A21	AA (A1–3,9,10–18)	Asp, Asp
		AB (A6, 21)	Asp, Asn
Metallo (E.C. 3.4.24)	M1-M51	MA (M1,2,4,5,9,13,30,36,48)	His, Glu, His (HEXXH)
		MB (M6–8, 10–12)	His, His/Asp (HGXXHXXGXXH/D)
		MC (M14)	His, Glu, His (HXXE/H)
		MD (M15)	His, His, Asp (HMYGHAAD)
		ME (M16, 44)	His, Glu, His (HXXEH)
		MF (M17)	Lys, Asp_3, Glu (NTDAEGRL)
		MG (M24)	Asp_2, His, Glu_2
		MH (M18,20,25,28,40,42)	His, Asp_3, Glu
		MX (M3,19,22,23,26,27,29,32,34–38, 41,43,45,47)	

(Lex A repressor), S26 (signal peptidase I), S41 (TSP protease), and S44 (tricorn protease). All known members of *clan SH* (catalytic triad: His, Ser, His) are endopeptidases from DNA viruses which are involved in virus prohead assembly. The clan includes only the family S21 (*Cytomegalovirus* assemblin). *Clan TA* with the catalytic residue Thr, Ser or Cys, and an α,β,α,β sandwich fold includes a number of peptidases whose only proteolytic activity is self-activation. Important families of this clan are T1 (proteasome), and S42 (γ-glutamyl transpeptidase).

Other families (*clan SX*) of serine peptidases including S14 (endopeptidase Clp), S16 (endopeptidase La), S18 (omptin), S19 (cell wall-associated endopeptidase of

Trichophyton), S34 (HflA endopeptidase), S38 (*Treponema* chymotrypsin-like endopeptidase), S39 (cocksfoot mottle virus endopeptidase), S43 (porin) cannot yet be assigned to clans, since neither the tertiary structure nor the order of catalytic residues are known.

The *cysteine peptidases* comprise the clans CA, CB, CC, CD, CE and CX. The last includes a number of other families of cysteine peptidases for which tertiary structures are unknown and virtually nothing is known about the specificity of the catalytic machinery.

The clan CA contains papain and its relatives. Papain was the first clearly studied cysteine peptidase. From the crystal structure of papain and a few closely related peptidases of the family C1, it could be concluded that the catalytic residues are Cys, His and Asn in that order of sequence. Further members of C1 are the cathepsine B, H, K, L and O, the dipeptidyl peptidase I, and glycyl endopeptidase. The C2 family contains various calpains, whereas streptopain belongs, to C10 ubiquitin C-terminal hydrolyse PGP 9,5 to C12, and the isopeptidase T to C19.

Clan CB contains viral "chymotrypsin-like" cysteine peptidases that process the viral polyproteins, and in clan CC are listed viral "papain-like" endopeptidases. The only family of clan CD (C14) comprises a number of cytosolic endopeptidases which cleave aspartyl bonds with high specificity. This family of caspases consists of ten members from which caspase-1 and caspase-3 are best known. The mature caspase-1, processed from a single-chain precursor by presumably autocatalytic cleavage of four aspartyl bonds, is a heterodimer of a 22 kDa heavy chain and a10 kDa light chain [19]. This peptidase was formerly known as interleukin 1β-converting enzyme (ICE) since it mediates, among other things, the processing of interleukin 1β at aspartyl bonds. Human caspase-3 is also a heterodimer consisting of the subunit p12 (11896 Da) and the subunit p17 (16617 Da) with a tertiary and quarternay structure similar to caspase-1 [20]. This peptidase appears to function in order to proteolytically inactive proteins which are involved in cellular repair and homeostasis during the effector phase of apoptosis.

Clan CE contains only the adenovirus endopeptidase [21]. A catch-all clan CX comprises all other families of cysteine peptidases which could not been classified up to now due to the lack of necessary data of structure and catalytic maschinery.

Aspartic peptidases have so far been described for all endopeptidases. Unfortunately, the tertiary structure has only been elucidated for four families. Endopeptidases of the family A1 consist of two lobes, with the active site between them. One lobe has been derived from the other by gene duplication. In the active site each lobe, with very similar three-dimensional structures, bears one Asp residue of the catalytic dyad. It is interesting to note that the crystal structure of retropepsin from family A2 of clan AA showed a single lobe with one catalytic Asp residue with structural similarity to one lobe of the pepsin from family A1. Retropepsin is only active as a homodimer forming the catalytic site between the two monomeric molecules. There is evidence that the peptidases of families A1 and A2 have evolved from a common ancestor. Unfortunately, a number of other families could not yet been assigned to any clan.

Metallopeptidases are allocated to eight clans. A couple of families could not yet be

assigned to these clans since, in particular, the metal ligands have not been biochemically characterized. Zinc-dependent metallopeptidases, both exopeptidases and endopeptidases, with the HEXXH motif are listed in the clan MA. The family M4 contains along with thermolysin, and elastase (*Staphylococcus*) well-known peptidases. The tertiary structure has been determined for members of this family showing a two-domain structure with the active site between the domains. The *N*-terminal domain contains the HEXXH motif and includes both α helices and β sheets as dominating structure elements and shows some similarities to the domain structure of clan MB.

In the *C*-terminal domain are five helices in a closed bundle. This characteristic fold is typical of thermolysin-like peptidases. Clan MC contains metallocarboxypeptidases which belong to only one family (M14) which is divided into the subfamilies A, B and C. Typical for this clan is that one zinc ion is tetrahedrally coordinated by a water molecule, two histidine and one glutamate residues. Clan MF includes aminopeptidases that require cocatalytic zinc ions for their enzymatic activity. The well-known leucyl aminopeptidase has a two-domain structure bearing the active site in the *C*-terminal domain. Whereas exopeptidases of clan MG require cocatalytic ions of cobalt or manganese, clan MH contains the third group of metallopeptidases that also require cocatalytic metal ions, but here these are all zinc ions. The third clan in which cocatalytic metal ions are necessary is clan MF with zinc or manganese. Only one catalytic zinc ion is required for peptidases of clans MA, MB, MC, MD and ME.

12.5.2.2
Importance of Proteolysis

Historically, enzymatic proteolysis has generally been associated with protein digestion. Therefore, the digestive peptidases of the pancreatic and gastric secretions are among the best characterized peptidases and much of the current knowledge of structure and function has been derived from investigations of those proteolytic enzymes. Activation of the pancreatic digestive enzymes is initiated by enterokinase, an enzyme secreted by the mucous membrane of the stomach. It converts some trypsinogen into active trypsin, which then activates all the proenzymes, including more trypsinogen. The function of the digestive proteases is merely to breakdown all the proteins they encounter.

Later, it became evident that peptidases play regulatory roles in a great variety of physiological processes[22, 23]. These include processing and molecular assembly of nascent polypeptide chains, and the processing of protein hormones, developing enzyme precursors to mature enzymes, fertilization, many other proteolytic processes important for cellular functions, and the regulation of the programmed cell death (apoptosis). The last is a mechanism that regulates cell number and is vital throughout the life of all animals. Apart from various biochemical events involved in apoptosis, the most fundamental one is the participation of members of the caspase family in both the initiation and execution phases of cell death. The mechanism of activation of the caspases constituting the different apoptosis-signaling complexes

can be explained by an unusual capability of the caspase zymogen to autopress to an active enzyme[23].

Proteolytic processing occurs in many different ways and is triggered by different proteases. Limited proteolysis is the key to this selectivity which depends on the accessibility of the scissile peptide bond to the acting peptidase and on its specificity. In this cases proteolysis is directed and limited to the cleavage of specific bonds in the target protein. A wide variety of prokaryotic and enkaryotic proteins are synthesized as larger pre- or pre-proforms. Some of these are biologically inactive and become activated upon limited proteolysis. Lysomal enzymes, mitochondrial proteins, membrane proteins, secreted proteins etc. undergo intracellular proteolytic maturation.

Various viruses code for specialized peptidases which are essential for virus assembly[24]. A couple of viral peptidases are interesting therapeutic targets. An extremely large number of publications have been dedicated to the aspartic peptidases, especially to the enzyme of the human immuno deficiency virus (HIV), which is a key target in the treatment of AIDS. HIV-1 protease (HIV-1 PR), more exactly named as human immunodeficiency virus 1 retropepsin (HIV-1 retropepsin; E.C. 3.4.23.16), has become the most thoroughly investigated system in the history of peptidases. The biological function of the retroviral peptidase is to cleave the polyprotein precursor into its constituent functional units such as the matrix, capsid, and nucleocapsid structural proteins of HIV to permit assembly. For this reason, the great interest in HIV-1 retropepsin has centered on the development of compounds that selectively inhibit the viral enzyme and not the related human aspartic peptidases. Useful principles of inhibition have been combined by several companies to produce antiviral compounds that have achieved approval from the Food and Drug Administration (FDA) in the USA (cf. review[25]). Despite the development of extremely strong and selective inhibitors which have been demonstrated to be effective in human trials one major problem remains: the extremely rapid development of forms of the virus that are resistant to the drugs containing the inhibitors.

Secretory proteins are usually synthesized as precursors bearing an aminoterminal extension. The signal peptide is removed co-translationally by signal peptidases during translocation across the membrane. In the next step precursors of protein hormones, growth factors and certain polycistronic precursor proteins are processed by specific enzymes. In contrast to consecutive zymogen activation consecutive pre-pro-cleavage reactions are regulated independently. The pathway of processing of many pre-proteins is known, but many of the maturation peptidases can not yet be characterized. For this reason, the application of molecular cloning techniques will be helpful in the near future for the sequence elucidation of pro-proteins as well as the cDNA and genomic sequences for maturation enzymes. The structure changes range from the relatively simple alterations in zymogen activation to more complex processing events in multidomain peptidase precursors, such as prothrombin or plasminogen. Generally, proteolytic processing induces intramolecular rearrangements required for the expression of biological response. Like blood coagulation, the complementary system is triggered by a signal that activates several consecutive zymogen activation reactions. This later system takes part in the immune reaction

directed against foreign organisms of tissues. Several components of the complementary system are serine peptidases.

Peptidases as integral components of cells have only been partly explored, e.g. lysosomal peptidases, granulocyte serine peptidases, membrane-bound peptidases, and enzymes of specialized tissues, such as the reproductive tract, skins, lens, muscle, pituitary, adrenals etc. Various ATP-dependent peptidases have been isolated.

The *proteasome* is a large multifunctional protease complex that degrades intracellular proteins. The name is derived from protease (*"protea-"*) and large particle (*"-some"*)[26]. This complex is an exception among peptidases as regards the nucleophilic residue and the general structure. Both in eukaryotes and archea, the proteasome is a multisubunit complex comprising four stacked rings each containing seven subunits (M_r ~20-30 kDa). In eukaryotes the *20S proteasome* (E.C. 3.4.99.46; also named multicatalytic proteinase, macropain and prosome) has the form of a hollow cylinder (length 148 Å, diameter 113 Å). It shows several different catalytic activities and contains 14 different but homologous subunits, whereas in archea there are just two different kinds of subunits, and the enzyme complex possesses only one catalytic activity.

In bacteria the proteasome is built up of two rings of six subunits. One of the two different subunits is related to the eukaryote and archaen proteasome subunits, the other is an ATPase. The *26S proteasome*[27, 28] (M_r ~2100 kDa) consists of the 20S proteasome and at least one other multisubunit regulatory protein known as PA700, 19S cap, μ-particle, ball, and ATPase complex. It was first found in extracts of rabbit reticulocytes by its capability to degrade ubiquitinated proteins in an ATP-dependent manner. Since this complex can also degrade various nonubiquitinated proteins the older designation ubiquitin-conjugate degrading enzyme (UCDEN) is probably inappropriate. The 20S proteasome subcomplex of the 26S proteasome containing multiple catalytic sites with distinc specificities is responsible for the whole proteolytic activity. In addition, the PA700 regulatory complex displays further enzymatic activities, such as ATPase activity, isopeptidase activity and seems to contain a substrate protein unfolding activity. The ATPase activity is necessary for assembly of the 26S proteasome from the 20S proteasome and PA700 subcomplexes and also for the degradation process. Since peptide bond hydrolysis is not energy dependent, the hydrolysis of ATP might be required for unfolding protein substrates and/or for translocation of the unfolded peptide substrate into the central channel of the proteasome. The proteasome is responsible for turnover of most cellular proteins in mammalian cells and the selective degradation of proteins with abnormal structures. Last but not least, the proteasome is involved in the production of antigenic peptides for presentation by MHC class I complexes. The generation of antigenic peptides seems to be performed by a specific subpopulation of proteasomes containing two or three subunits encoded in the major histocompatibility complex.

Considerable attention has been paid to a group of intracellular serine peptidases associated with granulocytes as well as leukocytes and mast cells as mentioned above. These peptidases are stored in granulas and released in response to inflammatory or allergic stimuli. Many of the peptidases are relevant to human health

and disease [29], some as natural components of the human body, and others because they are important in species which provide us with food, or cause diseases.

In oder to understand proteolytic activity in biological processes, knowledge of the contribution of the natural peptidase inhibitors to the regulation of the activity is essential [30]. Inhibitors are as diversified as the proteases themselves. Generally, they can be divided into two main classes: (a) active site-specific low-molecular-mass inhibitors, and (b) naturally occuring protein peptidase inhibitors. Examples of the first group are the serine peptidase inhibitors diisopropyl phosphofluoridate (DFP) and phenylmethanesulphonyl fluoride (PMSF). Both react with the active site serine. Many of the naturally occuring peptidase inhibitors, isolated from animal, plant and bacterial organisms, behave as pseudosubstrates. They combine essentially irreversibly with the active site and are converted into a modified form in which a peptide bond, related to the primary substrate specificity of the peptidase, is cleaved.

Of special physiological interest are inhibitors which react with mammalian plasma serine peptidases, especially those involved in blood coagulation. In principle, such inhibitors have both protective and regulatory functions. Approximately 10% of the nearly 200 proteins in blood serum are peptidase inhibitors. The α_1-proteinase inhibitor secreted by the liver, for example, inhibits leukocyte elastase which is thought to be part of the inflammatory process. Furthermore, special variants of this inhibitor with reduced inhibiting power are associated with pulmonary emphysema. The latter is a degenerative disease of the lungs which results from the hydrolysis of its elastic fibers. Interestingly, certain plants release peptidase inhibitors in response to insect bites in order to inactivate the digestive enzymes of the attacking insect.

Peptidases are valuable tools in the study of the primary and higher-order structure of proteins [31]. Proteolysis of proteins for sequence analysis and peptide mapping can be carried out according to different strategies [32]. Based on the extent of proteolytic reaction, it is allowed to reach completion or it is prevented from reaching completion. In the first case the products constitute an equimolar set of peptides whose composition will not be influenced by further digestion with same enzyme. Depending on the restriction imposed by the primary specificity of the peptidase used, a protein will be fragmented to varying degrees. The fragments can subsequently be separated and sequenced. Combining these data with sequence data of other overlapping sets which are generated with different peptidases allows the reconstruction of the sequence. If proteolysis is prevented from reaching completion a different set of data is obtained. Inhibition or removal of the peptidase are desirable interventions to determine the initial cleavage products.

Furthermore, peptidases are also structural probes of conformation of soluble proteins [33]. Although X-ray crystallography [34] and two-dimensional NMR [35] are the methods of choice for the determination of the three-dimensional structure of globular proteins, some weaknesses of these techniques demand alternative methods even if these will provide structural information at a lower level of resolution. For example, limited proteolysis can be used to probe the structure and the dynamics of proteins in solution, which provide experimental data that are easy to obtain and complement well those results derived from the techniques mentioned above. The

goal of investigations are soluble proteins in their native or near-native states. Limited proteolysis occurs in this case at the level of only one or very few peptide bonds which leads to the formation of "nicked" proteins. This species of proteins consists of rather large fragments which remain associated in a stable and often also functional complex. Usually, a nicked protein is much more labile than the native form. Therefore, the unfolding leads to a suitable substrate for an extensive proteolytic degradation to small peptides, and further proteolysis is much faster in comparison with the initial peptide bond cleavage at the level of the native protein. Consequently, in this case, during proteolysis the intact protein and small proteins are present in the incubation mixture, without intermediate sized products. In the case where nicked proteins are sufficiently stable, they may resist further extensive proteolytic degradation and can be isolated and characterized.

It is assumed that the limited proteolysis phenomenon derives from the fact that a specific polypeptide chain segment of the compact, folded protein substrate is exposed and flexible so that it can fit the active site of the appropriate peptidase for an efficient and selective limited hydrolysis. There is no doubt that enhanced chain flexibility or segment mobility is the key feature of the site of peptide bond hydrolysis demonstrated by a clear-cut correlation between sites of proteolytic attack and sites of enhanced chain flexibility. The present availability of automatic, efficient and sensitive techniques of protein sequencing and, particularly, the recent dramatic advances of mass spectrometry [36] in the analysis of peptides and proteins, allows a more systematic use of the limited proteolysis approach as a simple first step in the elucidation of structure-dynamics-function relationships for novel proteins which are only available in minute amounts.

Since a growing number of newly discovered peptidases are specifically expressed in single tissues, especially, at low expression levels or often only at certain development stages, it is very complicated to isolate the enzymes in sufficient quantities using classical biochemical procedures. Therefore, the only alternative is the cloning and expression of these peptidases. In addition, recombinant techniques allow directed structural alterations in order to program mechanistic or functional features. Peptidases can be expressed in most of the developed expression systems (yeast, viral, bacterial, insect cells and mammalian). It is not usually easy to predict which expression system is the method of choice. For functional expression of recombinant peptidases various examples have been presented [37].

Last but not least, it should be mentioned that a couple of peptidases have industrial importance. In particular, since subtilisins have a broad substrate specificity and are highly stable at neutral and alkaline pH they are of considerable industrial interest as protein-degrading additives to detergents. These reasons combined with their large data base make subtilisins attractive for protein engineering. Extensive engineering studies have been carried out on the *Bacillus* subtilins and more than 500 site-directed mutants have been produced to alter specific enzyme properties, such as pH profile, thermal stability or substrate specificity (see e.g. references [37–39]).

12.5.3
Formation of Peptides

12.5.3.1
Tools for Peptide Synthesis

Although the origins of peptide chemistry are usually traced back to the early 20th century when Emil Fischer obtained the simplest dipeptide glycyl-glycine by cleavage of the appropriate diketopiperazine, the first peptide bond in a chemical laboratory was synthesized by the young Theodor Curtius in the laboratory of Hermann Kolbe at Leipzig University in 1881. Despite the fact that Emil Fischer with co-workers in Berlin made basic contributions to peptide synthesis, the productive epoch of peptide chemistry began some decades later in the1950s. Wieland and Bodanszky[40] have written an excellent account of the history of peptide synthesis.

Peptides belong to an increasingly important class of bioactive molecules in physiology, biochemistry, medicinal chemistry and pharmacology. They act as hormones, neurotransmitters, cytokines, growth factors etc. However, it is not only naturally occuring physiologically relevant peptides that are the subjects of interest. Peptide analogs possessing agonist or antagonist activity are also useful tools in investigations when searching for suitable drugs. Radiolabeled analogs and molecules bearing affinity labels have been applied for the characterization and isolation of receptors. Furthermore, peptides are useful as substrates of peptidases, kinases, phosphatases and special transferases in investigations on enzyme kinetics, and mechanisms of action. In the preparation of polyclonal and monoclonal antibodies peptides play an important role as synthetic antigens, and epitope mapping using synthetic peptides has been developed as a valuable approach for the identification of specific antigenic peptides for the preparation of synthetic vaccines, and also for the determination of protein sequence regions which are important for biological function. In addition, the design of small peptide mimetics of protein function or structure, and the development of various peptidomimetics in drug development are further goals in peptide chemistry. In particular, in the last ten years the number of known peptides has doubled and besides the development of efficient chemicals for peptide synthesis methods, the field of peptide and protein chemistrry has been opened up to molecular biology and genetic engineering.

The classical chemical peptide synthesis is a synthesis in a homogeneous solution[41–46]. Even in the 1950s this approach had started to gain industrial importance followed by the solid-phase technique in the early 1960s, invented by the Nobel laureate Bruce Merrifield[47–50]. The most fundamental time-consuming operations in chemical peptide synthesis (sometimes not free from undesirable side reactions) are the selective protection, and after synthesis the deprotection of the α-amino function, the carboxyl group and the various side chain functionalities of trifunctional amino acids. Despite the development of numerous efficient protection methods based on chemical techniques, the whole process is rather slow as all intermediate products have to be purified and characterized after each reaction step. The formation of each peptide bond requires the activation of the carboxylic acid function of the carboxyl moiety.

An important point in selecting a coupling method is its degree of safety from racemization, since all synthetic operations carried out at a center of chirality have this permanent risk. Therefore, the synthesis of peptides with a multitude of chiral centers continues to be a formidable chemical effort. The existence of more than 150 chemical variations for peptide bond formation indicates that an ideal coupling method does not exist, e.g. a fast procedure without racemization or other side reactions to realize quantitative coupling of equimolar amounts of the carboxyl and amine components. There is no doubt that the use of well known strategies and the application of activation methods with well established safety steps to protect from racemization in simple model systems does not assure the loss of optical purity during a multitude of coupling steps in the synthesis of medium-sized and long peptides. For the chemical peptide synthesis in homogeneous solution, which still plays an important role in the production of large quantities of peptides for pharmaceutical use, highly skilled personel are required. In this manner multi-kilogram quantities or even tons of peptides consisting of the range of 2–30 amino acid residues can be produced.

Since peptides for research purposes are usually required in only mg to g amounts, the time-saving solid-phase peptide synthesis method[48–50] can be used. The strategy is in principle similar to that in solution, with the difference that there is no need for isolation of the intermediate products. As the growing peptide chain is synthesized on a suitable resin the whole procedure lends itself to automation. The drawback is that every reaction step at the resin has to be forced to give an almost 100% yield. In practice, this cannot be accomplished, with the consequence that the desired product must be isolated from a mixture of side-products by the final, normally HPLC, purification procedure which is sometimes difficult to perform and also expensive. Peptides of up to ~ 50 amino acid residues are now readily accessible using stepwise solid-phase procedures[50].

An alternative for the preparation of larger polypeptides and proteins is the biotechnological production (genetic engineering, recombinant DNA technology,) in bacteria, yeast, or cultured mammalian cells[51–53]. In principle, this is an economic way to produce peptides of more than 50 amino acid residues and even small proteins with complicated glycosyl or other groups attached to amino acid side chains. Compared with the problems connected to the chemical synthesis strategies, recombinant techniques provide quite a different set of problems. Whereas the principle of the expression of a gene in host cells through the normal biosynthetic and genetic machinery of the host cell using a suitable expression vector is relatively simple, putting this technique into practice poses some problems: Selection of the appropriate expression strategy, and the host cell system as well as the optimal vector system, the control of the stability of mRNA and also of the translated protein, isolation and purification of the product, scale-up, downstream processing etc.

The development of cloning vectors which propagate in eukaryotic hosts, e.g. yeast or cultured animal cells, has in particular eliminated many of the problems associates with the synthesis of eukaryotic proteins. It should be noted that post-translational processing may also vary among different eukaryontes. It is an advantage that shuttle vectors are available that are capable of propagating in both

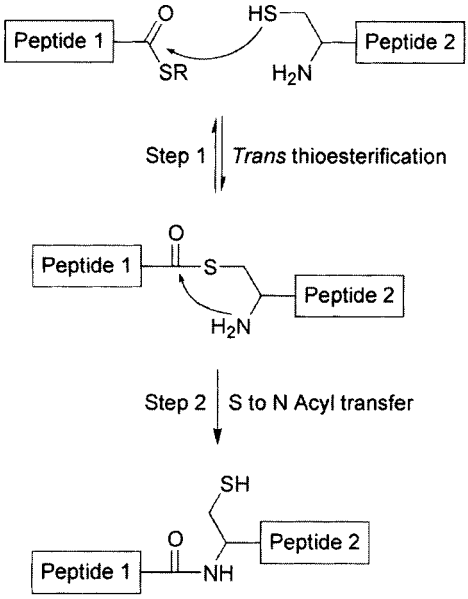

Figure 12.5-6. Principle of native chemical ligation according to Dawson et al.[55].

yeast and *E. coli* and thus transfer genes between these two cell types. Recombinant protein production is of great medical, agricultural, and industrial importance[54]. For example, human insulin, human growth factor, erythropoietin, various types of colony-stimulating factors, blood clotting factors are typical examples of recombinant proteins which are in routine clinical use.

Despite the fact that heterologous expression of recombinantly cloned genes is by far the most commonly employed method of to engineering proteins this approach is only applicable to naturally occurring amino acids. This limitation is in principal overcome by unnatural amino acid mutagenesis[54] and some other chemistry-driven approaches. Among the various chemical ligation methods the so-called "native chemical ligation"[55] has proved to be a useful route to fully synthetic proteins[55, 56–60]. As shown in Fig. 12.5-6 this procedure relies on the reaction that occurs between a peptide fragment possessing an essential *N*-terminal cysteine residue (peptide 2; Fig. 12.5-6), which can be expressed in principal using recombinant DNA procedures, and a second peptide fragment possessing an α-thioester group (peptide 1; Fig. 12.5-6). In an initial intermolecular, chemoselective reaction a thioester-linked intermediate is formed (step 1) which spontaneously rearranges *via* $S \rightarrow N$ acyl transfer to the final amide-linked product (step 2). The rearrangement step corresponds mechanistically to an intramolecular $S \rightarrow N$ acyl transfer reaction described by Wieland et al.[61] in 1953.

Pulling together protein splicing (for a review see reference[62]) and native chemical ligation led to "expressed protein ligation" (EPL)[63] or also termed "intein-mediated protein ligation (IPL)[64]. As shown in Fig. 12.5-7 the protein fragment of interest is expressed in *E. coli* as an intein-CBD (chitin binding domain) fusion protein. The chitin binding domain allows protein affinitity purification using chitin

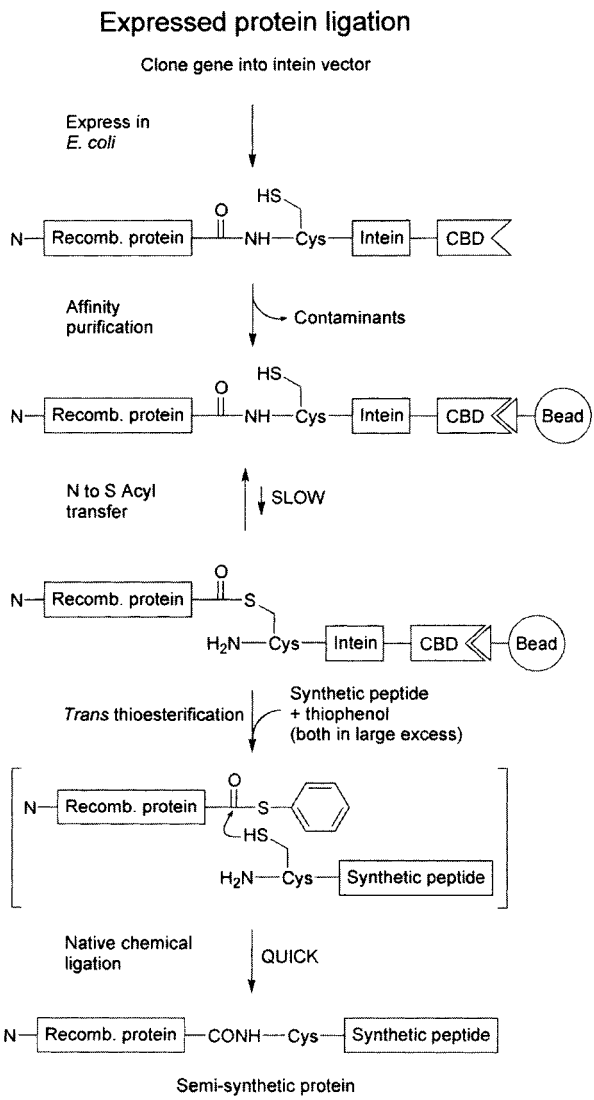

Figure 12.5-7. Principle of expressed protein ligation according to Muir et al.[63].

beads. The necessary expression vector is commercially available. The $N \rightarrow S$ acyl transfer results in a thioester-linked intermediate. In the next step a large excess of a suitable thiol agent (for example thiophenol) generates, by *trans*-thioesterification *in situ*, the protein α-thioester which reacts quickly with the simultaneously added synthetic amine component. The latter has to bear an *N*-terminal cysteine residue. Customized peptides containing *N*-terminal cysteine residues are available from a variety of sources. There is no doubt that the extension of the native chemical ligation to EPL led to significant progress in protein semi-synthesis[65], despite the remaining requirement of an *N*-terminal cysteine residue in the amine fragment to

be coupled. Apart from these advantages it must keep in mind that direct reaction of a thiophenyl ester with the amine component could result in partial epimerization of the *C*-terminal amino acid residue of the protein α-thioester.

12.5.3.2
Choice of the Ideal Enzyme

Enzymes have become valuable tools in medium to large-scale synthetic organic chemistry[66–70]. Owing to the fact that hydrolases possess a wide substrate spectrum and do not usually need cofactors for their catalytic function, they are at present the enzymes most widely used as biocatalysts in preparative organic chemistry. Among the hydrolases the huge family of peptidases plays an important role in various processes of proteolysis as shown above.

Unfortunately, a universal C - N ligase with a high catalytic efficiency for all possible combinations of the 21 proteinogenic amino acids both as *C*- and *N*-terminal amino acid residues, respectively, in fragments to be coupled could not be developed during evolution. Such heavy demands on specificity could not even be solved by nature. Therefore, protein biosynthesis has been developed as a step-wise strategy starting with the *N*-terminus of the growing peptide chain and catalyzed by the ribosomal peptidyltransferase. Limited proteolysis of the biosynthesis precursor molecules and posttranslational modifications provide the bioactive peptides and proteins. In nature the peptide bond formation is accomplished on the ribosome and takes place via the transfer of a peptidyl residue from the peptidyl-tRNA in the ribosomal P site to the amino group of aminoacyl-tRNA in the A site. Despite intensive investigations in recent years, the nature and the basic mechanism of the peptidyltransferase reaction within the ribosome is largely unknown. According to recent studies from the Nobel laureate Thomas R. Cech and coworkers[5] an *in vitro* selected ribozyme is capable of catalyzing the same type of peptide bond formation as a ribosome, e.g. its sequence and secondary structure seems to be strikingly similar to the "helical wheel" portion of 23S rRNA implicated in the activity of the ribosomal peptidyltransferase. These results provide evidence for the feasibility of the "RNA world" hypothesis by demonstrating that RNA itself is capable of catalyzing peptide bond formation.

Furthermore, from these findings the idea that the rRNA has a catalytic function in the ribosomal peptide bond formation is supported. It can be assumed that the selection of the individual aminoacyl-tRNA for the A site is mostly attributed to the specificity of the appropriate aminoacyl-tRNA synthetase together with the specific codon-anticodon interactions, whereas the 23 S rRNA participates in catalyzing peptide bond formation but without side-chain specificity for the amino acid esterified to the tRNA's 3'-terminal nucleoside. In comparison with the prerequisites for specificity of peptidases the peptidyltransferase seems to be an old unspecific ribozyme in accordance with its function in evolution as precursor to extant life.

It is of interest to note that in a recent paper a possible mechanism for peptide bond formation on ribosome without the mediation of peptidyltransferase has been proposed[71]. These authors assume, by analysis of the energetics using a semi-

empirical method for the formation of a cyclic intermediate, that the peptide bond formation through the tetrahedral intermediate in an *S*-configuration may not need assistance from an enzyme or ribozyme. From the tetrahedral intermediate a cyclic intermediate will be formed, where the 2'-OH of the ribose sugar of the P-site tRNA is a member of the ring, which produces a free tRNA and a tRNA attached to a planar peptide unit. Since the free 2'-OH group of the peptidyl-tRNA was proposed to be involved in peptide bond formation, it has been argued that the appropriate tRNA may be acting as a biocatalyst (enzyme or ribozyme).

Even in the case it should be possible to separate ribozyme activity from the ribosome or to isolate an *in vitro* selected ribozyme that can catalyze the same type of peptide bond formation as a ribosome, however such a biocatalyst seem does not to be suitable for simple practical use rather than using a chemical coupling reagent. In principle, this conclusion is also valid for the nonribosomal poly- or multienzymes which are involved in the biosynthesis of peptide antibiotics [72]. Up to now, they have only found application in the synthesis field of cyclosporin, gramicidin S, special β-lactam antibiotics and analogs.

At the end of this short assessment only those enzymes that usually act as hydrolases catalyzing the cleavage of peptide bonds remain to be discussed. The fundamental suitability of peptidases for catalyzing the formation of peptide bonds is based on the principle of microscopic reversibility that was predicted by van't Hoff in 1898 [73]. On the last page of his contribution he had proposed the basic idea of peptidase-catalyzed formation of the peptide bond, as follows:

"Die Frage ist berechtigt, ob.... auch nicht das Trypsin imstande ist, unter Umständen, durch die Gleichgewichtslage gegeben, Eiweiss zu bilden aus den Spaltprodukten, die es selber bildet".

The concept of van't Hoff of the equilibrium constant of a reversible chemical reaction, along with the function of a catalyst (including biocatalysts) for accelerated achievment of the equilibrium according to Ostwald [74], is the theoretical background of enzyme-catalyzed peptide synthesis. However, about 40 years elapsed before the first experimental proof of van't Hoff's prediction became evident through the first clear-cut peptidase-catalyzed synthesis of an amide bond carried out by Bergmann and Fraenkel-Conrat [75].Before this approach gained any industrial importance another 40 years had elapsed, and in recent decades considerable efforts have been made to find the optimum conditions for peptidase-catalyzed peptide synthesis as can be seen in various reviews [76–98].

12.5.3.3
Principles of Enzymatic Synthesis

As shown in Fig. 12.5-8 the equilibrium of a peptidase-catalyzed reaction is normally shifted to the thermodynamically more stable cleavage products. In contrast to proteolysis, the peptide bond formation is a two-substrate reaction and requires not only a specificity-dependent insertion of the carboxyl component into the *S*-subsites of the active site, but also an optimal binding of the amine component in the S' region. To shift the equilibrium in favor of fragment product formation various

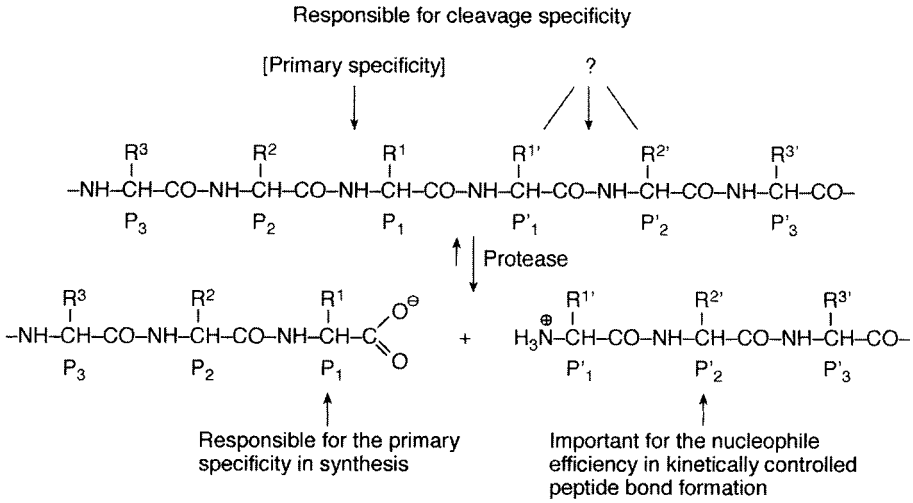

Figure 12.5-8. Peptidases function *in vivo* as hydrolases rather than as ligases.

manipulations are necessary which also differ mechanistically. The approaches to peptidase-catalyzed peptide bond formation are generally classified into basic strategies (see below) according to the type of carboxyl component used. In the equilibrium-controlled approach the carboxyl component bears a free carboxyl group as shown in Fig. 12.5-9, p. 826, whereas in the kinetically controlled approach the carboxyl component is employed in a slightly activated form, mainly as an alkyl ester. Both strategies are fundamentally different due to the energy required for the conversion of the starting components into the peptide products. Before interpreting the two mechanisms in more detail some general considerations of reversing proteolysis must be discussed.

12.5.3.3.1 General Manipulations in Favoring Synthesis

Looking at the equilibrium for the reversal of proteolysis, under normal conditions the equilibrium is shifted towards the hydrolysis products. For example, a synthesis of a dipeptide from its constituent free amino acids is, from the energetic point of view a very unfavorable process because of considerable increase in the free enthalpy involved. Under these circumstances it is not possible to accomplish peptide bond formation by simple reversal of hydrolysis, even using high concentrations of the starting amino acid zwitterions. Energetically more favorable is the reaction of an anion and a cation using an N^{α}-protected amino acid as a *carboxyl component* and a C^{α}-blocked amino acid as an *amine component*, respectively. According to the underlying thermodynamic principles, the outcome of peptide synthesis in aqueous solution depends on (a) the value of the equilibrium constant, (b) the ionization constants of the selectively protected starting compounds and (c) the initial concentrations of the ionized and nonionized forms of the carboxyl and amine component.

The thermodynamic parameters only allow statements relating to the free enthalpy change between the start and the end of the reaction, e.g. the equilibrium of the reaction. Only the velocity with which the equilibrium is reached depends on the catalytic action of the enzyme used.

According to the law of mass action the product yield is proportional to the starting component concentration. Using the least expensive starting component in excess, manipulations described in the following make it possible to transform the other starting component almost quantitatively into product.

The *formation of insoluble products* is a useful way of shifting the equilibrium towards synthesis.The reaction medium must be designed so that both starting components on the left-hand side of the equation are soluble in the medium while the peptide product on the right-hand side is insoluble. Under these conditions the product is continuously removed from the reaction medium by precipitation and sometimes an almost quantitative product yield can be obtained. A second way of reversing the proteolysis reaction can be performed by *product extraction*, a concept quite close to the solubility-controlled process of precipitation. The reaction is carried out in a biphasic system where the product is much more soluble in the organic phase and is continuously removed from the aqueous phase where the starting components and the enzyme are soluble. In both approaches to product removal the benefits of the appropriate organic solvent must be taken into consideration, which will be discussed later. Last but not least, in special cases the formed product can be separated from the equilibrium by *molecular traps*, where the desired product will removed by specific complex formation, as demonstrated, for example, in the course of clostripain-catalyzed fragment condensation of the ribonuclease (RNase) fragments 1–10 with 11–15 using RNase S(21–124) as a trap[99].

12.5.3.3.2 Equilibrium-controlled Synthesis

This equilibrium-controlled or thermodynamic approach (see below) represents the direct reversal of proteolysis. Consequently, all peptidases, independent of their mechanisms, can be used. Apart from this advantage the necessary high enzyme requirement and the low reaction velocity are drawbacks of this approach.

Preceding the conversion, determined by K_{con}, is an ionization equilibrium K_{ion}:

$$RCOO^- + H_3\overset{+}{N}R' \overset{K_{ion}}{\rightleftharpoons} RCOOH + H_2NR' \overset{K_{con}}{\rightleftharpoons} RCO-NHR' + H_2O \qquad (1)$$

If the water concentration is taken into the equilibrium constant Eq. (2) is obtained:

$$K_{syn} = K_{ion} \cdot K_{con} = [RCO-NHR'](\![RCOO^-]\,[H_3\overset{+}{N}R']\!)^{-1} \qquad (2)$$

The reaction medium, especially the pH, determines the constants for a given pair of reactants. To obtain an equilibrium that is shifted in favor of peptide product formation the ionization equilibrium must be manipulated. One efficient method is the addition of water-miscible organic solvents to the aqueous reaction mixture thereby lowering the dielectric constant of the medium, reducing the acidity of the carboxyl group, and to a lesser extent the basicity of the amino group of the nucleophilic amine component[100, 101]. The use of biphasic systems (for a review

see reference[102]), e.g. solvent systems consisting of an aqueous phase and a nonmiscible phase (nonpolar organic solvents) does not damage the enzyme since it is localized in the aqueous phase. Under ideal conditions the reactants diffuse from the organic phase into the aqueous phase and after peptide bond forming step the product diffusses back into the organic phase. Only the insufficient solubility of the reactants in nonpolar organic solvents limits the general application of the biphasic approach, particularly for the condensation of longer segments.

For the direct reversal of catalytic hydrolysis of peptides, discussed in this chapter, the term equilibrium-controlled approach should be preferred. Because of the thermodynamic control of both equilibria in Eq. (1) the reversal of proteolysis is often denoted as a thermodynamic approach. In order to increase the product yield of this endergonic process various manipulations are required. In addition to those mentioned above, reverse micelles[103], anhydrous media containing minimal water concentrations[104], water mimics[105], and reaction conditions promoting product precipitation as discussed in first part of this chapter are often employed.

12.5.3.3.3 Kinetically Controlled Synthesis

In contrast to the equilibrium-controlled approach the peptidase-catalyzed kinetically controlled peptide synthesis (for a review see reference[85]) needs much less enzyme, the reaction time to reach maximal product yield is significantly shorter, and the product yield depends both on the properties of the enzyme used and the substrate specificity. Kinetic control means that the product appearing with the highest rate and disappearing with the lowest velocity would accumulate. Whereas the equilibrium-controlled approach ends with a true equilibrium, in the kinetic approach the concentration of the product formed goes through a maximum before the slower hydrolysis of the product becomes important. The product will be hydrolyzed if the reaction is not stopped after the acyl donor ester is consumed and true equilibrium is allowed to be reached.

In Fig. 12.5-9 both approaches are compared schematically. The kinetic approach (b) requires the use of an acyl donor ester as a carboxyl component and is limited to

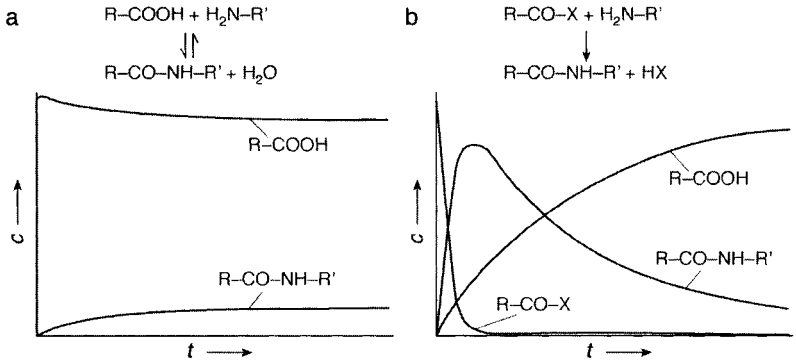

Figure 12.5-9. Comparison of the equilibrium (a) and the kinetically controlled approach (b) of peptidase-catalyzed peptide synthesis.

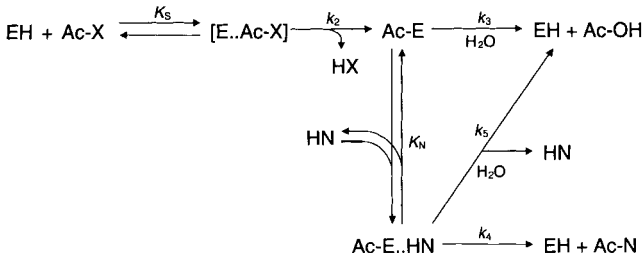

Figure 12.5-10. Kinetics of peptidase-catalyzed acyltransfer reaction. EH = enzyme, Ac-X = acyl donor ester (carboxyl component), HN = nucleophile (amine component), HX = leaving group of the acyl donor ester, Ac-OH = hydrolysis product, Ac-N = peptide product; E..Ac-X = enzyme-substrate complex (Michaelis complex), Ac-N..HN = acylenzyme-nucleophile complex.

peptidases which rapidly form an acylenzyme intermediate, e.g. serine and cysteine peptidases. The peptidase acts as a transferase catalyzing the transfer of the acyl moiety to the amino acid- or peptide-derived amine component. Specifically, the acylenzyme reacts, in competition with water, with the nucleophilic amine component to form the peptide bond. The ratio of formation of aminolysis and hydrolysis products is of decisive importance for successful preparative peptide synthesis. Figure 12.5-10 describes the kinetics of the peptidase-catalyzed acyl transfer reaction. First, the acylenzyme is formed via the Michaelis-Menten complex, which binds the amine component to the acylenzyme. The resulting acylenzyme-nucleophile complex can undergo aminolysis as well as hydrolysis. The acyl transfer efficiency of the peptidase for the corresponding substrates is determined by the ratio of the aminolysis and hydrolysis product formed, which is also denoted as selectivity.

12.5.3.3.4 Prediction of Synthesis by S' Subsite Mapping.

Serine and cysteine peptidases are not perfect acyltransferases. Therefore, it is useful to have a method for the prediction of the outcome of the kinetically controlled peptide synthesis. In order to get a simple efficiency parameter we decided to introduce the partition value p[106] analogous with the definition of the Michaelis constant according to Eq. (3), where P_2 = Ac-OH, P_3 = Ac-N, and N = HN.

$$\frac{v_H}{v_A} = \frac{d[P_2]}{d[P_3]} = \frac{p}{[N]} \tag{3}$$

The p value corresponds to the nucleophile concentration at which hydrolysis and aminolysis of the acylenzyme proceeds with the same velocity. The advantage of p is that the definition is not based on a particular kinetic scheme. Furthermore, p allows a rapid estimation of the yield of any acyl transfer reaction. A concentration of the nucleophilic amine component $[N] >> p$ is necessary for peptide formation in high yield. Assuming an equilibrium between the acylenzyme and the acylenzyme-

nucleophile complex, Eq. (4) and (5) can be derived from Fig. 12.5-10 for the velocities of hydrolysis and aminolysis of the acylenzyme, where E = EH, EA = Ac-E, A = Ac-X, and EAN = Ac-E..HN.

$$v_H = [EA]k_3 + [EAN]\frac{k_5}{K_N} \tag{4}$$

$$v_A = [EAN]\frac{k_4}{K_N} \tag{5}$$

Eq. (6) results from combination Eq. (4) and (5).

$$p = \frac{[N]k_5}{k_4} + \frac{K_N k_3}{k_4} \tag{6}$$

It follows from Eq. (6) that a linear correlation between the partition value p and the nucleophile concentration is obtained. The quotient k_5/k_4 corresponds to the ratio of hydrolysis and aminolysis of the EAN complex whereas the term $k_N k_3/k_4$ is a measure of the nucleophile efficiency.

The partition value p can be determined by differnt methods[107–109]. In the presence of a large excess of nucleophile ($[N] >> [A]_0$) the decrease in the nucleophile concentration during the reaction course can be ignored. Under these conditions $v_H/v_A = [P_2]/[P_3]$. The determination of p can be established out from the product ratio obtained by HPLC analysis according to Eq. (7).

$$p = \frac{[P_2][N]}{[P_3]} \tag{7}$$

In the preparative application of acyl transfer reactions, however, a large excess of the nucleophile is not useful because a complete turnover of both reactants is desired. For this reason, we developed the determination of p from the integrated rate equation[109] according to Eq. (8).

$$\frac{[P_2]}{[P_3]} = \frac{k_5}{k_4} + K_N\frac{k_3}{k_4}\frac{\ln([N]_0/([N]_0-[P_3]))}{[P_3]} \tag{8}$$

A plot of $[P_2]/[P_3]$ versus $\ln([N]_0/([N]_0 - [P_3]))/[P_3]$ gives a straight line with the slope $K_N\ k_3/k_4$ and an intercept with the y axis at k_5/k_4. Since this method permits the determination of p under the conditions employed in preparative peptide synthesis it should be useful for the optimization of the reaction conditions.

An understanding of the molecular interactions between the acylenzyme and the attacking nucleophilic amine component allows an optimization of the acyl transfer efficiency. The efficiency of the nucleophilic attack of the amine component depends essentially on an optimal binding within the active site by S' - P' interactions (Fig. 12.5-11). Consequently, more information on the specificity of the S' subsites of serine and cysteine peptidases are useful, which can be obtained by systematic acyl transfer studies using libraries of nucleophilic amine components. According to the definition of the p value (see above) small values of p indicate high S' subsite specificity for the appropriate amine component in peptidase-catalyzed acyl transfer reactions.

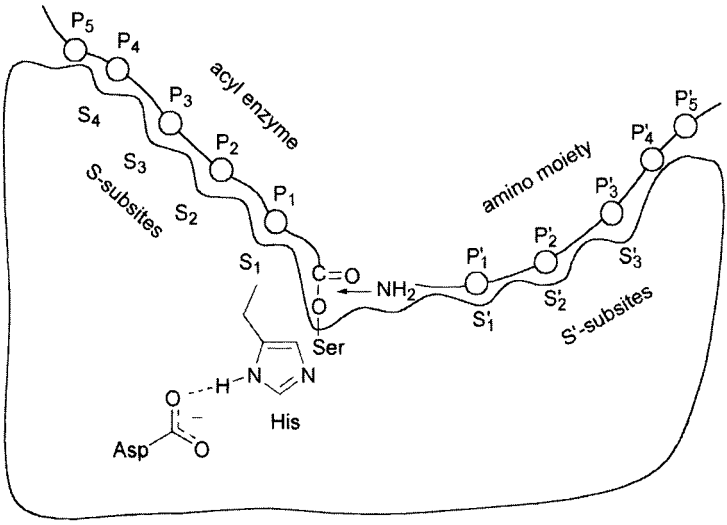

Figure 12.5-11. Schematic representation of subsite-substrate interactions in the course of the acyl transfer from the acylenzyme to the nucleophilic amine component catalyzed by a serine peptidase.

We have studied a couple of different serine peptidases (for a review see reference[85]), the cysteine peptidases papain[110] and clostripain[111, 112], respectively, and the prolyl endopeptidase from *Flavobacterium meningoseptum*[113], and have determined *p* values for various series of nucleophilic amine components. Apart from clostripain none of the enzymes under investigation catalyzed acyl transfer to nucleophilic amine components with P'_1 = Pro or D-amino acids. The efficiency of chymotrypsin-catalyzed acyltransfer decreases in the order of positively charged > aliphatic > aromatic > nagatively charged P'_1 side chains. The specificity of chymotrypsin for P'_1 = Arg and Lys is attributed to electrostatic interactions between these side chain moieties and Asp^{35} and Asp^{36} in the active site. A statistical analysis of proteolysis data confirmed that chymotrypsin possesses a specificity for peptide bonds bearing Arg or Lys at the P'_1 position, whereas Leu-Asp bonds of proteins were cleaved by this enzyme considerably less frequently than one expects from the frequency of occurence of this peptide bond[114]. Our results confirm this statistical evaluation exactly. Furthermore, remarkably chymotrypsin prefers arginine residues at the P'_1 and P'_3 positions, which offers an interesting option for using chymotrypsin in the sense of a restriction peptidase for peptide-catalyzed processing of recombinant proteins (cf. Fig. 12.5-27).

The selectivity of the *S'* subsites of different peptidases is reflected by the broad range of data obtained as shown for simple amino acid amides in Table 12.5-3. The values demonstrate the preference of basic and hydrophobic P'_1 residues for chymotrypsin and also for papain. In the case of chymotrypsin the strongly basic side chain of arginine amide gives rise to a higher efficiency than all other nucleophiles. Despite the difficulties in catalyzing Xaa-Pro bonds, we have studied the clostripain-catalyzed acyltransfer using a large number of proline-containing peptides as well as

Table 12.5-3. Comparison of *p* values of selected amino acid amides H-Xaa-NH_2 in acyltransfer reactions catalyzed by various serine and cysteine peptidases according to Schellenberger and Jakubke[85].

Enzyme		*p*	(mM)	
Xaa	Arg	Leu	Val	Met
Endoproteinase Glu-C V8	> 500	16	117	64
Endoproteinase Glu/Asp-C	30	132	n.d.	382
Chymotrypsin	0.11	4.2	6.7	3.3
Trypsin	66	72	130	12
Elastase	16	62	69	34
Papain	1.3	0.41	3.9	1.5

Ala-Xaa dipeptides and amino acid amides[111, 112]. The efficiency of clostripain-catalyzed acyltransfer, using Bz-Arg-OEt as the acyl donor to amino acid amides decreases in the order Leu > Lys > Gly > Arg > Gln > Ser > Pro > Thr > Ala > Asn > Asp > Glu. *S'* subsite mapping using an Ala-Xaa library led to the result that clostripain prefers P'_2 residues with positively-charged side chains, followed by proline, whereas negatively-charged side chains of Asp and Glu are weak nucleophilic acceptors. In the pentapeptide series, containing only one proline residue, the efficiency decreases in the order Pro-P'_3 > Pro-P'_2 > Pro-P'_1. Surprisingly, PAPAG, PPAAG and PF-NH_2 act as very weak nucleophilic acceptors. The variety of different conformations of proline-containing peptides should be the reason for the extreme differences in enzyme-nucleophile interactions.

12.5.3.3.5 What Approach Should be Preferred?

As mentioned above, the equilibrium-controlled approach has the advantage that all peptidases can be used. However, the high enzyme requirement and the low reaction velocity are serious drawbacks. Owing to the endergonic process the reaction conditions must be manipulated in order to increase the product yield. The addition of high concentrations of water-miscible organic solvents to decrease the p*K* value of the carboxyl component very often decreases the catalytic activity of the peptidases. Furthermore, by carrying out equilibrium-controlled synthesis in aqueous media using reactants with unprotected side chain functions, the specificity-determining amino acid residue should again not occur in the segments to be coupled.

In the kinetic approach, the serine or cysteine peptidase rapidly reacts with a suitable acyl donor ester to form the acylenzyme intermediate, which can be deacylated competitively by the added nucleophilic amine component and water. The ratio between aminolysis and hydrolysis of the acyl donor ester is of great importance for the outcome of the synthesis route.This selectivity is essentially determined by the *S'* subsite specificity of the enzyme as shown above. To establish an optimum synthesis strategy, it is useful to know the basic kinetic parameters for the reaction course, in particular those obtained by *S'* subsite mapping are of great importance for planning and optimization of the enzymatic synthesis.

Depending on the specificity of the peptidase used, pH and solvent conditions, the

peptide product formed in the kinetic approach is quite stable since the amidase activity of most enzymes is lower than the esterase activity. In addition, the esterase activity can be positively manipulated by varying the type of leaving group, as shown later. For preparative peptide synthesis such a manipulation is very important as it allows complete conversion of the acyl donor ester before the product is hydrolyzed. There is no doubt that the course of kinetically controlled protease-catalyzed peptide synthesis can be influenced more efficiently than the equilibrium approach.

Although the kinetic approach should be preferable, the decision must depend on the overriding total synthesis concept. The largest industrial scale application of the equilibrium approach is probably the enzymatic synthesis of Z-Asp-Phe-OMe, the precursor of the peptide sweetener aspartame[115]. The best known use of trans-peptidation technology is the large scale conversion of porcine insulin into human insulin by trypsin[116] or *Achromobacter lyticus* protease[117].

12.5.3.4
Manipulations to Suppress Competitive Reactions

The most important factors which limit the widespread routine application of peptidases in kinetically controlled peptide synthesis are undesired hydrolysis of the acyl donor ester and proteolysis of both the starting segments to be coupled and the final peptide product, respectively (Fig. 12.5-12). An elimination or minimization of these undesired reactions can be performed by various manipulations concerning the reaction medium, the enzyme and the substrate as well as on mechanistic features of the process. In particular, an efficient leaving group of the acyl donor ester can provide high reaction rates in combination with a decreasing danger of possible proteolysis of the starting segments and the final product.

12.5.3.4.1 **Medium Engineering With Organic Solvents**

In peptidase-catalyzed peptide synthesis the solubility of the starting components dramatically influences the course of the synthesis. From the ideal medium, water, the spectrum of solvents ranges from water-miscible organic solvents and aqueous-organic biphasic systems to monophasic organic solvents with trace amounts of

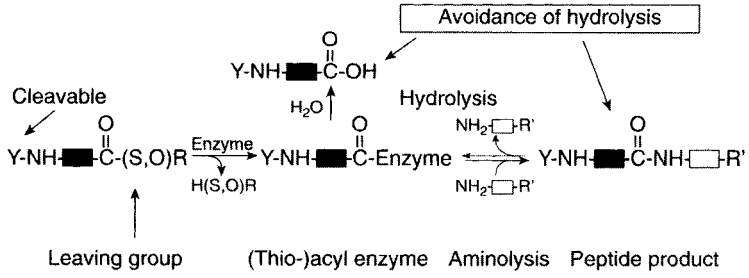

Figure 12.5-12. General course of the kinetic approach to fragment condensation catalyzed by serine or cysteine peptidases.

Reaction medium	Advantages	Drawbacks	Alternatives
Water			
	ideal medium for enzymes	poor solubility for partially protected reactants	use of solubilizing protecting groups
	optimal ecological conditions	kinetic approach only promotion of hydrolysis	
Water/Water-Miscible Organic Solvents			
	increased reactant solubility	reduzed enzyme activity	use of chemically or genetically modified enzymes
	promoting equilibrium-controlled approach	difficult product isolation	
Water/Water-Nonmiscible Organic Solvents	**[Biphasic Systems]**		
	prevention of enzyme activity	higher enzyme requirement	use of chemically or genetically modified enzymes
	easy product isolation	limitation of reactant solubility lowering of velocity	
Monophasic Organic Solvents	prevention of hydrolysis	reduced enzyme activity	use of chemically or genetically modified enzymes
	no solubility problems of partially protected reactants	change of stereo- and regiospecificity	
	adjusting media between chemical and enzymatic strategies	higher enzyme requirement	

Figure 12.5-13. Influence of the reaction medium on peptidase-catalyzed peptide synthesis.

water necessary for the catalytic activity of the enzyme (Fig. 12.5-13). Not only for ecological reasons, but water should be the preferred reaction medium for enzymatic processes, since it is *in vivo* the medium of choice for enzymes anyway.

Solubilizing Protecting Groups

These are the only alternative way of bypassing the poor solubility of most amino acid-derived starting components, and synthesis of peptides can only be performed if one or both reactants bears such a solubility-promoting group. A successful synthesis of kyotorphin (Tyr-Arg) in a continuous large scale procedure using highly solubilizing N^{α}-protecting groups was carried out by Fischer et al.[118]. They used maleyl (Mal-, 3-carboxyacryloyl-), a group which increases both the solubility of the tyrosine ethyl ester as well as the activity of chymotrypsin. This procedure was performed with concentrations of Mal-Tyr-OEt of up to 1.5 mol L^{-1} and an equimolar

concentration of H-Arg-OEt. A 72.7 mol procedure resulted in 12 kg of the diacetate of Tyr-Arg which corresponds to an overall yield of 50.4% including protecting group removal, purification by ion exchange chromatography, and final product isolation by spray drying. Further large scale procedures using solubilizing protecting groups were carried out by Flörsheiner et al.[119] and Hermann et al.[120]. It was also reported that carboxypeptidases are capable of coupling *N*-terminally unprotected amino acid esters (50 mM) to unprotected amino acids as well as amino acid derivatives (0.2–1.5 M) in one step at room temperature in aqueous solution[121]. This synthesis principle is more generally applicable to other esterolytic endopeptidases or lipases[122–124]. The reduced stereoselectivity allows synthesis of D,L-dipeptides in higher yields than the corresponding L,L-dipeptides[125]. The chymotrypsin-catalyzed coupling of H-Phe-OMe with nucleophilic amine components in a frozen aqueous state[126] starting from lower acyl donor/nucleophile ratios should be mentioned as an interesting alternative, and enzyme-catalyzed synthesis in frozen-aqueous systems will discussed later in more detail (see Sect. 12.5.3.4.2).

Water/Water-Miscible Organic Solvent Systems

Such systems promote the solubility of partially protected starting compounds and increase the p*K* value of the carboxyl component in equilibrium-controlled processes thereby promoting this synthesis course. However, reduced enzyme activity in the presence of high portions of organic solvents and difficulties in product isolation are sometimes serious drawbacks. Despite these limitations such media with a small organic solvent content are preferred in enzymochemical peptide synthesis. The application of more stable immobilized enzymes as well as chemically or genetically engineered enzymes offers advantages in cases of high contents of organic solvents, as will discussed below.

Biphasic Aqueous/Organic Systems[127, 128]

These have been developed as an alternative to water/water-miscible organic solvents systems. This approach leads to preservation of enzyme activity and allows simple product separation, an advantage which is counteracted by prolonged reaction times where additional partition equilibria are most likely to be the rate-determing steps. The general use of biphasic systems is mostly limited by the solubility of the starting components in the nonpolar organic phase. This alternative to the use of water-miscible organic solvents has been used with various peptidases and good yields were obtained using no more than two equivalents of the nucleophilic amine component (for a review see reference[102]).

Synthesis in Reversed Micelles[129, 130]

This is principially very similar to the approach discussed above. After adding small amounts of water and a surfactant to a hydrocarbon, the polar ends of the surfactant form a sphere which contains the water. Since the lipophilic group of the surfactant is facing outside into the surrounding hydrocarbon, the reverse structure of a normal micelle is formed. Liposome-assisted selective polycondensation of amino acid and peptides shows an interesting continuation along this line[131].

Monophasic Organic Solvents[132]

The ultimate way of preventing undesired hydrolytic side reactions in the course of peptide synthesis is offered by these solvents. Trace amounts of water between approximately 0.3 to about 1% are necessary to maintain the catalytic activity of the enzyme. Although it has been generally assumed that higher concentrations of water-miscible organic solvents significantly reduce the catalytic activity of the peptidases, few papers have demonstrated successful enzymatic peptide synthesis performed in some hydrophilic organic solvents, such as aliphatic alcohols and acetonitrile[133–136]. Generally, enzyme specificities change dramatically in organic solvents. Higher enzyme requirement and reduced rates should be noted. It is interesting to mention that peptidases also catalyze esterification and transesterification reactions in organic solvents when the appropriate alcohol is added.

Chemically or Genetically Modified Peptidases

They provide a useful alternative for peptide synthesis in high concentrations of organic solvents since they are more stable than the native enzymes. Various possibilities for modification are known.

Immobilized Enzymes

Such enzymes can be used in a very simple way for enzymatic peptide synthesis as first reported by Jakubke and coworkers[128, 137–139] at the beginning of the 1980s. The effort involved in immobilizing an enzyme is mostly compensated for by the possibility of its repeated use. Immobilized biocatalysts have almost the same efficiency as the native enzymes. The peptidase is covalently linked or adsorbed to an insoluble gel or resin. The water content in these systems plays an important role in modulating the catalytic properties of the immobilized peptidase. The presence of water molecules on the enzyme is required in order to retain the catalytic activity. The measurement and control of the thermodynamic water activity is necessary to quantify the water effect on enzyme activity and the intrinsic influence of other variables such as support, solvent and educts[140, 141]. The advantage of these systems have been demonstrated in the synthesis of various biologically active peptides[141, 142].The effect of water-miscible aprotic solvents on kyotorphin synthesis catalyzed by immobilized chymotrypsin was studied by Lozano et al.[143] Of special technical interest are the continuous synthesis of the aspartame precursor Z-Asp-Phe-OMe with thermolysin immobilized on amberlite XAD-7 in a plug flow type reactor[144] and the conversion of porcine insulin into human insulin catalyzed by *Achromobacter lyticus* protease I immobilized on SiO_2-polyglutamic acid[145].

Solvent-Modified Enzymes

These are named as enzymes which are modified, for example, with polyethylene glycol (PEG) allowing synthesis in monophasic organic solvents as described, e.g. for chymotrypsin[146, 147], papain[148] and thermolysin[149]. Using PEG-modified enzymes in monophasic organic solvents undesired proteolytic reactions can be almost completely eliminated. However, owing to the solubility properties the use of hydrophobic organic solvents makes the application for the synthesis of longer

peptides very complicated and often impossible. Insoluble cross-linked chymotrypsin[150] can be obtained using glutaraldehyde concentrations several times higher in contrast to the procedure for soluble polymeric preparations of chymotrypsin[151]. Insoluble cross-linked chymotrypsin was used in a medium with 60% (v/v) dimethylformamide (DMF) for successful synthesis of short peptides. High amounts of powdered suspensions of peptidases in DMF have been used for peptide synthesis[152]. An very interesting synthesis approach has been described using *cross-linked enzyme crystals* (CLECs)[153, 154].

Chemically Modified Enzymes

Enzymes are often prepared with the aim of reducing the peptidase activity with some of the esterase activity remaining, thus preventing the hydrolytic cleavage of peptide bonds[86]. Methyl-chymotrypsin (MeCT) obtained by *N*-methylation of His^{57} shows a significant change in the enzymatic catalysis. MeCT is less active than native chymotrypsin by a factor 10^4 to 10^5 but it is virtually without any peptidase activity[155]. Owing to the low activity more activated cyanomethyl ester is used instead of methyl ester. Subtilisin can also be changed to an acyltransferase via modification of the active site serine to cysteine (thiol subtilisin with low amidase activity[156]) or seleno subtilisin[157]. Successful synthesis of various L,D-dipeptides using [$Met(O)^{192}$]chymotrypsin[158] were carried out as well as the synthesis of Ac-Tyr-OEt from Ac-Tyr-OH and ethanol catalyzed by hexyl-chymotrypsin in a biphasic system[159].

Genetically Engineered Enzymes

They have elevated solvent tolerance and also owing to the lowering amidase activity have been successfully used for synthetic purposes[160]. Enzyme engineering describes a range of techniques from deliberate chemical modification as shown above to remodeling a wild-type enzyme by gene technology. The aim of engineering peptidases to generate peptide ligases by conversion of serine and cysteine peptidases via site-directed mutagenesis, is to make enzymes more stable and favor aminolysis rather than hydrolysis. Using multiple site-directed mutagenesis subtilisin can be converted into a mutant which allows kinetically controlled synthesis to be performed in the presence of high concentrations of DMF. An ingenious combination of chemical and enzymatic steps should promote the progress in peptide and protein synthesis as was demonstrated with subtiligase, a double mutant of subtilisin BNP'. This variant was prepared by protein design and used in a further total synthesis of Ribonuclease A (RNase A)[161] by combining solid-phase synthesis for fragment synthesis and enzymatic coupling of these fragment to form the protein (cf. 12.5.3.7.2, p. 856). The selection for improved subtiligases by phage display results in the identification of two new mutants that increased the activity of subtiligase[162].

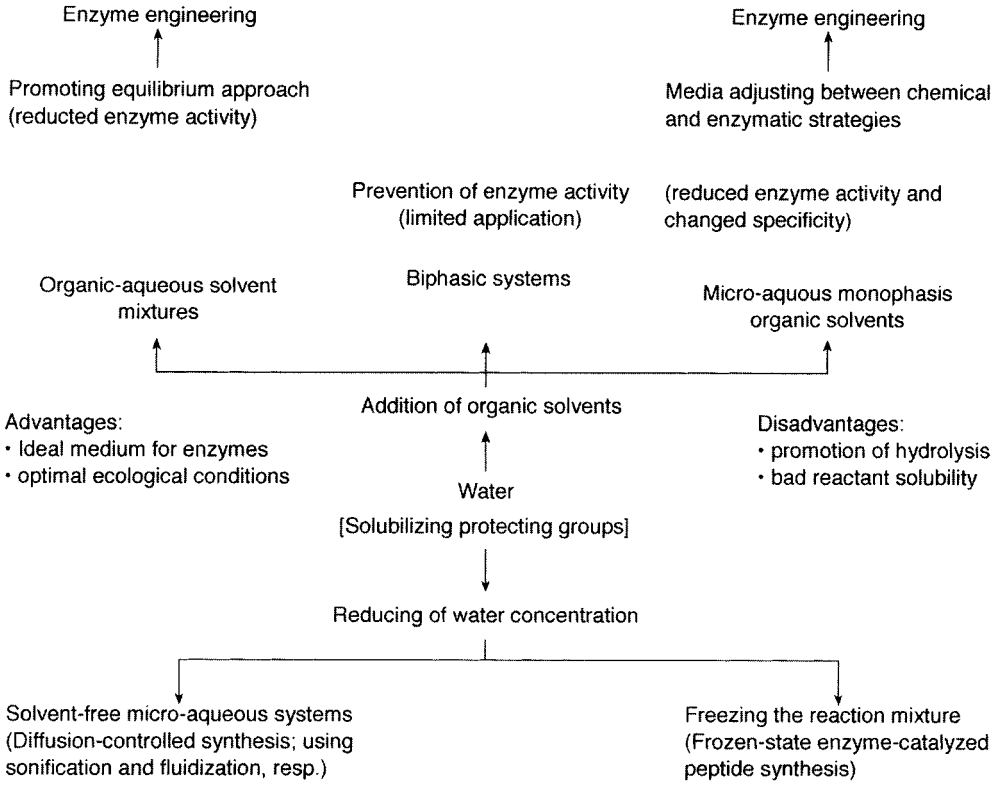

Figure 12.5-14. Extended approaches to medium engineering in enzymatic peptide synthesis [96].

12.5.3.4.2 **Medium Engineering by Reducing Water Content**

Competitive reactions in enzymatic peptide synthesis are, as mentioned above, mainly undesired hydrolysis of the acyl donor ester in the kinetic approach, and undesired proteolytic side reactions in both the starting components in fragment condensation as well as the final product. It can be demonstrated that side reactions of these types can be largely, but not completely, avoided by synthesis in organic solvents of controlled water activity. However, since the main drawbacks caused by organic solvents are enzyme deactivation and changes in specificity, which can only partly be improved by enzyme engineering, new strategies (Fig. 12.5-14) in reducing the water concentration without substitution by organic solvents have been described (for a review see reference [91]).

Enzymatic Peptide Synthesis in Frozen Aqueous Systems

This is based on observations by Grant and Alburn [164] that trypsin-catalyzed hydroxylaminolysis of amino acid esters was favored over hydrolysis in frozen reaction mixtures (for a review see Hänsler and Jakubke [163]). In 1990 Schuster et

al.[165] first reported on the influence of freezing on peptidase-catalyzed kinetically controlled peptide synthesis. The peptidase is added to the reactants precooled to 0 °C in a polypropylene tube and immediately inserted into liquid nitrogen. After 20 s the tube is transferred into a cryostat at – 15 °C or similar temperature. Amino components that are considered to be inefficient nucleophiles in enzymatic synthesis at room temperature gave substantially higher yields in frozen reaction mixtures. Later these results could be explained on the basis of the so-called freeze-concentration model[166] and were confirmed by other investigators[167].

In frozen aqueous systems the endopeptidase chymotrypsin is capable of acting as a reverse carboxypeptidase catalyzing coupling of free amino acids as amino components[168]. Various amino acids were acylated under catalysis of chymotrypsin starting from 2 mM Mal-Phe-OMe and 50 mM (50% as free base) of the appropriate amino acids at – 25 °C in unexpectedly high yields (% given in parentheses): Met (75), Val (58), Ser (52), Ile (35), Thr (30), Asn (29), Leu (26), Lys (60). Tougu et al.[169] described similar results on coupling Mal-Tyr-OEt with free amino acids. The surprising catalytic behavior of chymotrypsin under frozen state conditions is demonstrated in Table 12.5-4. N^{α}-unprotected amino acid esters as well as dipeptide esters, even containing unusual amino acids, can be coupled in frozen aqueous systems in high yields indicating both reverse aminopeptidase and dipeptidylpeptidase activities. Furthermore, cysteine proteases, with the exception of clostripain, were capable of catalyzing peptide synthesis in high yields using amine components with low efficiency at room temperature in frozen reaction mixtures. The specific properties of peptide synthesis in frozen solutions such as changes in specificity observed in serine and cysteine peptidase-catalyzed reactions strongly suggest that factors other than concentration of the reactants are probably involved in yield-enhancement by freezing. This assumption is supported by investigations reported by Jakubke et al.[171] who determined the amount of unfrozen water in frozen samples at – 15 °C using the ^{1}H-NMR-relaxation time technique and obtained an apparent concentration factor of 50. Synthesis experiments carried out under these concentration conditions at room temperature gave substantially lower yields com-

Table 12.5-4. Comparative model peptide synthesis catalyzed by chymotrypsin in frozen aqueous systems and at room temperature.

Acyl donor	Amino component	Peptide Ice	Yield (%) 25 °C	Reference
Mal-Tyr-OMe	H-β-Ala-Gly-OH	79	< 2	[165]
Mal-Tyr-OMe	H-D-Leu-NH_2	73	10	[166]
Mal-Phe-OMe	H-Lys-OH	60	0	[168]
H-Tyr-OEt	H-Lys-OH	71	0	[169]
H-Phe-OMe	H-Leu-NH_2	94	52	[170]
H-4-fluoro-PheOMe	H-Leu-NH_2	90	47	
H-2-naphtyl-Ala-OMe	H-Leu-NH_2	93	55	
H-Leu-Phe-OMe	H-Ala-Ala-OH	88	5	[171]
H-Asp-Phe-OMe	H-Ala-Ile-OH	91	23	
H-Gly-Phe-OMe	H-Ala-Ile-OH	85	23	

pared with frozen reaction mixtures and, therefore, could not simulate the reaction conditions in ice.

In addition to the freeze-concentration effect, a catalytic role for ice crystals, a favorable orientation of substrate and biocatalyst, the markedly lower dielectric constant of ice compared with water, and the high proton mobility in ice, have been discussed as further factors that possibly influence reactions in frozen systems. In summary, the reverse action of hydrolases provides an attractive alternative to the chemical synthesis of peptides but this approach could also be verified for the synthesis of oligosaccharides and oligonucleotides using glycosidases and ribonucleases, respectively[163].

Peptidase-catalyzed Synthesis in Solvent-free Micro-aqueous Systems

Such systems show a second route to reducing water concentration without substitution by organic solvents. This interesting development allows the application of reaction systems with partly undissolved reactants and is based on an extensive theoretical treatment of the equilibrium position described by Halling et al.[172]. The principle of a solid-to-solid conversion is illustrated graphically in Fig. 12.5-15 and selected examples of its experimental implementation are illustrated in Table 12.5-5. The application of solid phase substrate pools combines the equimolar (or nearly equimolar) supply of starting compoments with high obtainable yields, easy work-up procedures and compatibility with chemical standard procedures. The key parameter for obtaining high product yields via acyltransfer reactions is the ratio of aminolysis and hydrolysis favored by high nucleophile concentrations. In combination with solid phase acyl donor pools, this approach allows an equimolar supply of starting materials without any addition of organic solvents. The synthetic potential of systems with partly unsolved reactants was proven by pilot scale synthesis of Z-His-Phe-OMe and the low calorie sweetener precursor of Z-Aspartame in the thermodynamic approach[173] and by kinetically controlled synthesis of enkephalin derivatives[174]. Furthermore, Halling and co-workers have studied the effect of water and

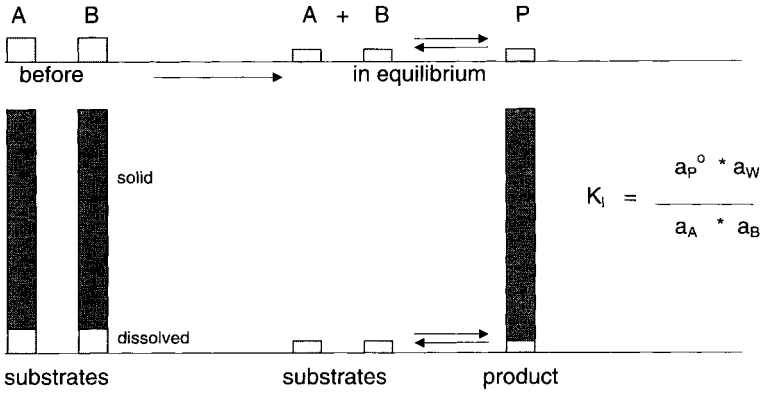

Figure 12.5-15. General principle of application of "equilibrium shift" towards the product by solid-phase substrate pools (botton) compared with synthesis starting from solution[172].

Table 12.5-5. Selected examples for peptide synthesis in water-based solid-liquid systems according to Eichhorn et al.[173] and Jakubke et al.[91].

Carboxyl component	Amine component	Peptide yield (%)	Time (h)	Enzyme
Z-Ala-OH	H-Leu-NH_2	95	0.5	Thermolysin
Z-Asp-OH	H-Phe-OMe	90	7	Thermolysin
Z-Gln-OH	H-Leu-NH_2	94	4	Thermolysin
Z-Phg-OH	H-Leu-NH_2	89	2	Thermolysin
Z-Ser-OH	H-Leu-NH_2	89	2.5	Thermolysin
Z-His-OH	H-Leu-NH_2	95	3	Thermolysin
Z-Phe-OH	H-Met-NH_2	88	24	Thermolysin
Ac-Tyr-OEt	H-Arg-NH_2	90	1	Chymotrypsin
Ac-Tyr-OEt	H-Gly-Gly-OH	63	2	Chymotrypsin
Z-His-Phe-OBzl	H-Arg-Trp-NH_2	95	2.5	Chymotrypsin
Z-Ser-OCam	H-His-ONb	85	2.5	Papain
Z-Gly-His-ONb	H-Lys-NH_2	90	1.5	Chymotrypsin
Z-Arg-His-ONb	H-Gly-NH_2	55	6	Subtilisin

enzyme concentration of thermolysin-catalyzed solid-to-solid peptide synthesis in detail[175] and reviewed the recently developed approach to enzymatic synthesis with mainly undissolved substrates at very high concentrations[176].

12.5.3.4.3 Substrate Engineering

In the case where undesired subsequent reactions occur during kinetically controlled synthesis it is of minor importance which bond is cleaved by the enzyme. These side reactions underline the issue that the specificity of the enzyme for the acyl donor ester does not lie sufficiently above its specificity for the peptide product. Since the sequence of the starting components cannot be changed, the only practical alternative to suppress such competitive reactions is to use a highly specific leaving group of the acyl donor ester. As a simple model peptide with a highly sensitive cleavage site for chymotrypsin Schellenberger et al.[177] used the chromogenic chymotrypsin substrate Mal-Leu-Phe-pNA (Mal = maleyl), which is formed by chymotrypsin-catalyzed coupling of Mal-Leu-OY with H-Phe-pNA. Table 12.5-6 shows that the leaving group moiety Y is of major influence on the reaction course. When Mal-Leu-OMe is employed as the carboxyl component, the velocity of the product cleavage reaches the rate of its formation after a short time. By using the

Table 12.5-6. Influence of the specificity constants of acyl donor esters on the yield of the chymotrypsin-catalyzed synthesis of Mal-Leu-Phe-pNA[a] starting from Mal-Leu-OY with varying leaving groups Y and H-Phe-pNA according to Schellenberger et al.[177].

Leaving group Y	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M ($M^{-1}\,s^{-1}$)	Reaction time (min)	Yield (%)
Me(Methyl)	120 ± 30	5.6 ± 0.4	4.7×10	10	~3
Bzl(Benzyl)	1.7 ± 0.4	5.4 ± 0.4	3.2×10^3	5	65
$Nb_{(4\text{-Nitrobenzyl})}$	0.38 ± 0.1	5.9 ± 0.4	1.5×10^4	5	80.5

a k_{cat}/K_M of Mal-Leu-Phe-pNA: $1.2\times10\ M^{-1}\,s^{-1}$ (Mal; maleyl).

more specific acyl donor esters (higher specificity constants k_{cat}/K_M) a clear product accummulation is attained. With Mal-Leu-ONb or ester of similar or higher specificity, starting components containing highly protease-labile cleavage sites can be coupled even in a homogeneous phase in high yields. According to this finding Bongers et al.[178] published a two-step enzymatic semisynthesis of the superpotent analog of human growth hormone releasing factor [deNH$_2$Tyr^1,D-Ala^2, Ala^{15}] GFR(1–29)-NH$_2$ from the amine component [Ala^{15}]GRF(4–29)-NH$_2$ and the carboxyl component deNH$_2$Tyr-D-Ala-Asp-OY (Y = Et or 4-NO$_2$Bzl, respectively) catalyzed by V8 protease and Glu/Asp-specific endopeptidase (GSE) from *Bacillus licheniformis*, respectively. Using the 4-nitrobenzyl leaving group compared with the ethyl moiety results in a higher yield without the undesired proteolytic side reactions. The state-of-the-art of substrate engineering is without doubt the substrate mimetic-mediated C - N ligation strategy which allows irreversible peptide bond formation and will be presented separately (see Sect. 12.5.3.6).

12.5.3.5
Approaches to Irreversible Formation of Peptide Bond

Despite the development of various possibilities to suppress competitive reaction, as shown in the preceding section, an absolute avoidance of proteolytic cleavage of the peptide bond formed cannot be guaranteed. The only alternative seems to be the use of biocatalysts that do not have the catalytic potential to hydrolyze peptide bonds.

12.5.3.5.1 Use of Nonpeptidases

Nonpeptidases are supposed to possess favorable prerequisites for the formation of peptide bonds because undesired proteolytic cleavages in the starting components and the product can be ruled out.

Enzymes involved in protein synthesis also possess potential, e.g. aminoacyl-tRNA-synthetase-aminoadenylate complex[179], the arginyl-tRNA: protein arginyltransferase[180], and nonribosomal poly- or multienzyme complexes[72] which require ATP or GTP to activate the carboxyl group of an amino acid, and seem to accept various amino acid nucleophiles for peptide bond formation. However, the application of these enzyme systems for generally practical peptide bond formation is rather limited.

Furthermore, lipases[181, 182] containing the catalytic triade typical for serine peptidases have been used for peptide synthesis as well as pig liver esterase[183, 184]. These enzymes accept both D- and L-amino acid derivatives as weak acyl donors or nucleophilic acceptors, but concerning the practical importance the situation is similar to the enzyme systems reported previously.

Particularly promising from the theoretical point of view seems to be the developments in catalytic antibodies. It could be established that antibodies raised against suitable transition state analogs are capable of catalyzing the formation of peptide bonds[185, 186]. At present the practical importance is rather low, but the development of more tailor-made catalytic antibodies for peptide bond formation could change the situation in the future.

12.5.3.5.2 Use of Proteolytic Inactive Zymogens

In 1994, it was firstly established that zymogens, the catalytically inactive precursors of various peptidases, can be used as biocatalysts for practically irreversible peptide bond formation[91, 96, 97, 187]. The capability of reacting slowly with site-specific reagents indicated that such reactions proceed via the formation of an acyl-zymogen intermediate[188, 189]. Although, the second-order rate of ester hydrolysis is 10^6–10^7 times slower than by the appropriate active enzyme, the deacylation rates of zymogen and active enzyme do not differ significantly. Therefore, it can concluded that the conversion of a zymogen to an enzyme should not be the activation of an inert zymogen, but the potentiation of catalytic activity intrinsic to the zymogen.

Based on this feature Jakubke's group has used the zymogens of the well studied serine peptidases trypsin and chymotrypsin, respectively, in peptide synthesis experiments, and has surprisingly observed catalysis of peptide bond formation by the zymogens trypsinogen and chymotrypsinogen. In several cases *S'* subsite mapping studies showed significant differences in the deacylation of the acyl enzymes compared with the corresponding acyl zymogens, based on acyl transfer to various peptide derivatives. Although the zymogens possess the same catalytic triad, which is necessary for the formation of the appropriate covalent acyl intermediate, the non-optimal formed substrate binding cleft prevents proteolysis. In particular, Gly^{193} is distorted and is not capable of forming a hydrogen bond to the carbonyl oxygen of the substrate which is necessary for the stabilization of the oxyanion-hole[190]. However, because of the high flexibility in this region, a principle oxyanion stabilization takes place, although not in an ideal manner. To confirm true zymogen catalysis it was essential to prove that the zymogen preparations were not contaminated with traces of the appropriate active enzyme. Based on the significantly different affinity of both enzyme and zymogen to the basic pancreatic trypsin inhibitor (BPTI) it was possible to analyze the esterase activity of the zymogen, which is an efficiency parameter used in estimating their peptide bond forming potential. Since the differences in k_{cat}/K_M cover a range of about 5 orders of magnitude, for general use of zymogen catalysis it is essential to improve the acylation rate.

The application of zymogens for irreversible fragment condensations was studied by coupling a synthetic tetrapeptide methyl ester with a recombinant 24-peptide according to the procedure of Cerovsky et al.[98]. A comparison of the coupling reactions (Fig. 12.5-16) was carried by dropping the acyl donor ester **1** into a solution of the amine component **2** and, alternatively, under batch conditions. The first way was chosen in order to minimize the undesired ester hydrolysis of **1** and, in addition, to manipulate a large excess of the amine component **2**. In this case 5.4 mg (0.002 mmol) of **2** was coupled with 4 mg (0.008 mmol) of **1** in the presence of 0.5 mg of chymotrypsinogen and resulted, after 400 min, in the complete conversion of the acyl donor ester in 60 % yield to the desired product **3**. The batch procedure led to a product yield of 52 %.

In order to avoid any undesired zymogen activation by limited proteolysis, e.g. of the Lys^{15}-Ile^{16} peptide bond in the case of trypsinogen, it would be useful to prevent this reaction by chemical means. The guanylation of trypsinogen by 1-guanyl-3,5-dimethylpyrazole causes a stable zymogen because of the conversion of all lysine

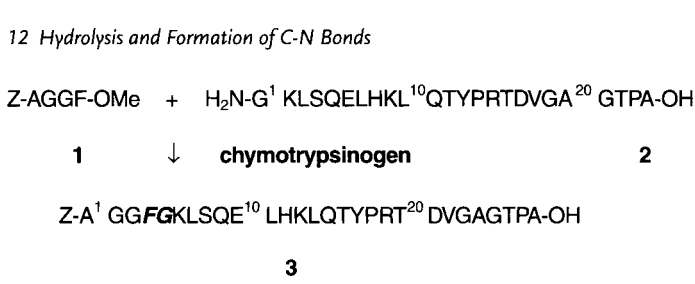

Figure 12.5-16. (4+24)-Fragment condensation catalyzed by chymotrypsinogen according to Cerovsky et al. (cf. reference[98]).

residues into homoarginine (Har), including the crucial Lys^{15}. Peptide synthesis with the guanylated zymogens led to very surprising results. Using dipeptides with a free carboxyl group as the amine components they are much more effectively accepted by the the guanylated species[91]. From molecular modelling studies it can be concluded that there is an interaction between the carboxyl group of the dipeptide with the only lysine within the active site (Lys^{61}). The conversion of Lys^{61} to homoarginine increases the pK of the side chain and therefore the basic character.

12.5.3.6
Irreversible C-N Ligations by Mimicking Enzyme Specificity[191]

The synthetic importance of peptidases as biocatalysts for peptide synthesis is undisputed due to a couple of advantages over pure chemical coupling methods. The mild reaction conditions and the high degree of regio- and stereospecificity guarantees both freedom from partial epimerization and that there is no need for temporary protection of side-chain functions. On the other hand, there are some serious drawbacks of the classical peptidase approach which has been discussed below in detail. Most important is the fact that the formed peptide bond formed can be cleaved in the course of the catalytic process by the same enzyme. There are no differences in the requirements of the specificity in both the peptide bond forming step and cleavage step, respectively. Since the specificity is manifested by the side-chain of the P_1 amino acid residue, e.g. Arg or Lys in the case of trypsin, an irreversible peptide bond formation seems not to be possible according to the classical concept of reversal of proteolysis.

In ribosomal peptide bond formation the mechanism is based on an acyl transfer of the acyl moiety from the peptidyl-tRNA (or fMet-tRNA at the start of the prokaryotic biosynthesis) located at the P site of the ribosome to the amino group of the aminoacyl-tRNA in the A site catalyzed by the side-chain unspecific ribozyme peptidyltransferase. Learning from nature our philosophy was that mimicking specificity is the only way to make the peptidase-catalyzed peptide bond forming step irreversible[192, 193, 197]. Since from the mechanistic point of view the kinetic approach with serine and cysteine peptidases is also an acyl transfer process, the idea arose of to transfering the specificity moiety of the P_1 amino acid side chain to the leaving group of the acyl donor ester. In this manner the enzyme should recognize the acyl donor ester. However, after the acylation of the enzyme the leaving group

with the specificity determinant is released from the enzyme with the consequence that the peptide bond formed cannot be cleaved by the enzyme due to the lack of specificity for recognizing this bond. In 1991 we were able to confirm this assumption by model peptide synthesis catalyzed by trypsin using various nonspecific N^{α}-protected amino acid 4-guanidinophenyl esters (OGp) as acyl donors and various amino acid and peptide derivatives as nucleophilic acyl acceptors[192, 193], and later extended by further examples from another group[194, 195]. At that time this type of acyl donor ester was named an inverse substrate according to time-dependent irreversible inhibitors of trypsin and trypsin-like peptidases, such as 4-amidino- and 4-guanidinophenyl esters which were found to be hydrolyzed by these peptidases virtually idependently of their acyl moieties[196, 197]. Although this fact was first published in 1973 by Wagner and Horn[196], very little was known about the basic mechanism of the hydrolysis of these inverse esters. In 1997 an extension of this new approach to irreversible peptide segment condensation with other peptidases was described and the term *substrate mimetics* was introduced by Bordusa et al.[197].

12.5.3.6.1 Mechanism of Substrate Mimetic Hydrolysis

The most striking structural differences of N^{α}-protected amino acid or peptide 4-guanidinophenyl esters compared with common peptide substrates are the nonspecific acyl residue and the highly specific leaving group. It was established by Bordusa's group[198] that all 4-guanidinophenyl esters, independently of structure and chirality of the acyl moiety, are hydrolyzed despite the lack of trypsin-specific acyl moieties, with the exception of the lysine derivatives (Table 12.5-7). This behavior is in contrast to common trypsin substrates. According to the familiar model, conventional trypsin substrates bind with their acyl residue to the *S*-binding site of the enzyme having the leaving group at the *S'*-subsite and the scissile bond between attacked by Ser^{195}.

Table 12.5-7. Steady-state kinetic parameters for the hydrolysis of Boc-Xaa-OGp by trypsin[a] according to Thormann et al.[198].

Ester (Xaa)	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M (M^{-1} s^{-1})
L-Ala	0.206	32.4	1.6×10^5
D-Ala	0.161	0.61	3.8×10^3
Gly	0.087	23.5	2.7×10^5
L-Leu	0.146	38.8	2.7×10^5
D-Leu	0.035	0.85	2.5×10^4
L-Gln	0.239	35.2	1.5×10^5
D-Gln	0.071	0.68	9.6×10^3
L-Phe	0.211	66.1	3.1×10^5
D-Phe	0.249	9.0	3.6×10^4
L-Glu	0.071	5.5	7.6×10^4
D-Glu	0.039	0.43	1.1×10^4
L-Lys	0.107	270	2.5×10^6
D-Lys	0.314	15.7	5.0×10^4

a Conditions: 25 mM Mops, pH 7.6, 100 mM NaCl, 5 mM $CaCl_2$, 25 °C; errors less than 15%.

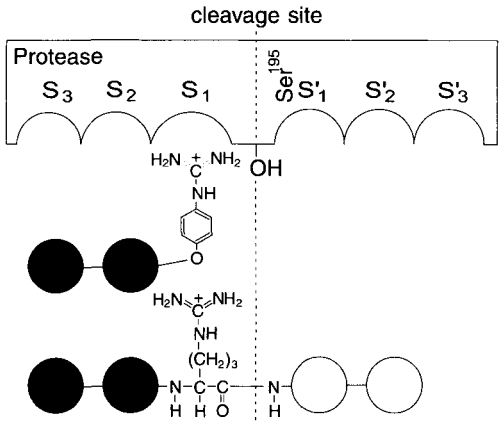

Figure 12.5-17. Schematic comparison of the binding of a peptide 4-guanidinophenyl ester and a common trypsin substrate to the active site of the enzyme according to the conventional binding model.

As shown schematically in Fig. 12.5-17 applying the same binding principles for the acyl moiety of the substrate mimetics leads to a catalytically unproductive binding. The acyl residue binds at the *S*-subsite of trypsin, but the scissile bond would be far away from the active site and, therefore, and cannot be attacked by Ser^{195}. However, docking calculations show that the specificity-bearing OGp group binds to the S_1-binding pocket like the side chain of L-arginine of commom peptide substrates. Surprisingly, this holds even for the substrates Boc-L-Arg-OGp and Boc-L-Lys-OGp despite the presence of the S_1 specific arginine and lysine residues, thus indicating a higher S_1-specificity for the 4-guanidinophenyl moiety. Indeed, all L- and D-substrate mimetics realize an arrangement in such a way that the scissile bond is very close to the hydroxyl group of the active Ser^{195}. Furthermore, the carbonyl group of the scissile ester bond of the appropriate substrate mimetic is located at exactly the same position as the carbonyl group of the scissile peptide bond between P_1-Lys^{15} and P_1'-Ala^{16} in the trypsinogen-BPTI complex. This implies a possible attack by trypsin, which was confirmed by the hydrolysis studies.

How does it work from the mechanistic point of view? Contrary to common trypsin substrates, the acyl residues of these enzyme-substrate mimetic arrangements bind to the *S'*-subsite of trypsin (Fig. 12.5-18). For this reason, all binding sites beyond S_1 are only of minor importance for the substrate mimetics. Furthermore, the acyl residues of the substrate mimetics do not reflect the specificity of the *S*-

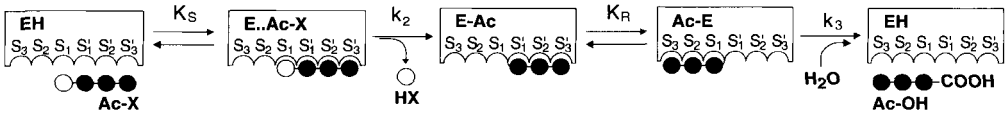

Figure 12.5-18. Schematic representation of the new extended kinetic model of peptidase-catalyzed hydrolysis of substrate mimetics according to Thormann *et al.*[198].
EH, free enzyme; Ac-X, substrate (substrate mimetic); [E..Ac-X], Michaelis-Menten complex; HX, leaving group; E-Ac, acyl enzyme intermediate located in S'-region; Ac-E, acyl enzyme intermediate located in S-region; K_R, rearrangement equilibrium constant; Ac-OH, hydrolysis product.

binding site of the enzyme. Since the direction of the peptide backbone chain is reversed, the *S'*-subsite specificity is also not reflected. Therefore, substrate mimetics show a unique specificity behavior.

The deacylation step, however, requires an unoccupied *S'*-subsite since water can only attack the acyl enzyme from this site without hindrance. Hence, the flipping acyl moiety acts like a "sliding window" within the active-site, spanning the primed and unprimed subsite regions. The extended kinetic model requires a rearrangement step between the two arrangements (E-Ac and Ac-E) of the acyl enzyme described by the equilibrium constant K_R (Fig. 12.5-18). From the experimental data of Table 12.5-7 it follows that D-configured substrates exhibit lower k_{cat} values, which might be related to lower K_R values. Exploring the dynamic behavior by molecular dynamics simulations of Boc-L-Ala-trypsin and Boc-D-Ala-trypsin indicated that the flip of the D-Ala complex to the *S*-subsite takes about 1.5 ns, much more than in the L-Ala complex (300 ps).

For an experimental study of the *S'*-subsite accessibility, *S'* mapping studies (cf. Sect. 12.5.3.3.4) are suitable. By their specific *S'*-binding capacity, peptide nucleophiles should be capable of pushing aside the acyl moiety from the S' region more efficiently than water. Therefore, the aminolysis of acyl enzymes bearing the acyl moiety in *S'* should proceed at higher rates compared with their hydrolysis. Indeed, from the mapping studies it follows that the *p*-values for the deacylation of Bz-D-Ala-trypsin are dramatically lower than for Bz-L-Ala-trypsin. Consequently, the experimental data of aminolysis also support this unique catalysis mechanism for substrate mimetics.

12.5.3.6.2 Cationic Substrate Mimetics

The N^{α}-protected amino acid 4-guanidinophenyl ester was the first example of substrate mimetics for Arg-specific peptidases used for irreversible peptide bond formation[192, 193]. Apart from the guanidino group linked at various aromatic and aliphatic spacers, also the amidino moiety is also suitable as a specificity-determing residue in the leaving group of cationic substrate mimetics[192–195]. After the basic studies with trypsin we could also establish that other Arg-specific peptidases such as thrombin and clostripain are suitable enzymes for peptide synthesis using cationic substrate mimetics[197]. Im particular, clostripain has been very useful in substrate mimetic-mediated fragment condensation. As shown in Fig. 12.5-19 the (3 + 5) fragment condensation provided a product yield of over 90% within a few minutes and the product formed remains unchanged after 72 h. The course of this synthesis clearly proves the irreversibility of this model C - N ligation.

For synthesis planning, clostripain has an additional decisive advantage due to the extremely low P_1' specificity for the *N*-terminal amino acid residue of the amine component. Firstly, Bordusa and co-workers[199] demonstrated impressively the capability of the cysteine peptidase clostripain as a biocatalyst for the synthesis of peptide isosteres. These authors have investigated the function of clostripain for acylating aliphatic noncyclic and cyclic amines varying in chain length and ring size using the trypsin standard acyl donor ester Bz-Arg-OEt. Furthermore, using a model

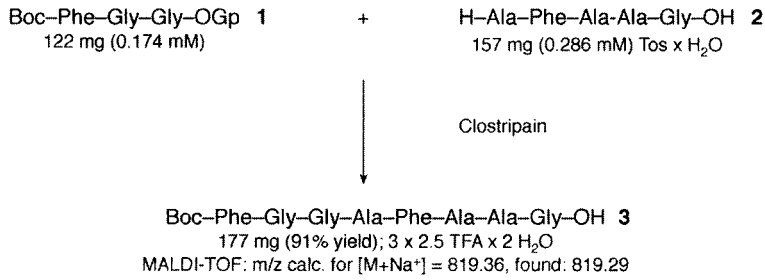

Figure 12.5-19. Clostripain-catalyzed (3 + 5) fragment condensation of Boc-Phe-Gly-Gly-OGp and H-Ala-Phe-Ala-Ala-Gly-OH[197]. Conditions: 50 mM HEPES-buffer, pH 8, 100 mM NaCl, 10 mM $CaCl_2$, 25°C, [Clostripain]: 1.6 μM.

substrate mimetic, clostripain was capable to catalyze the reaction with noncoded and non-amino acid-derived amines. The results of these investigations indicate that the substrate mimetic approach may extend outside of peptide synthesis.

In a recent paper Bordusa's group presented a novel enzymatic approach to the synthesis of carboxylic acid amides using substrate mimetics and clostripain as a biocatalyst[200]. This unexpected peptidase-mediated approach to the coupling of non-coded and non-amino-acid-derived amines with pure organic esters could only be realized by the combination of the substrate mimetic strategy with the use of clostripain that possesses a broad tolerance towards amines. Selected examples of the clostripain-catalyzed coupling of Bz-β-Ala-OGp and the 4-guanidinophenyl ester of 4-phenylbutyric acid (Pbu-OGp) with various amino acid amides and peptides are summarized in Table 12.5-8. Furthermore, the broad tolerance of clostripain toward non-coded amino acids and even simple amines, such as aliphatic, aromatic, or substituted amines including unnatural amino acids, and diamines as acyl acceptors is demonstrated by the results of appropriate syntheses compiled in Table 12.5-9. The substrate mimetic approach has opened a new range of synthesis applications beyond peptide synthesis offering efficient and selective organic amide bond formation under extraordinarily mild reaction conditions.

Table 12.5-8. Clostripain-catalyzed coupling of 4-guanidinophenyl esters of 4-phenylbutyric acid (Pbu-OGp) and benzoyl-β-alanine (Bz-β-Ala-OGp), respectively, with various amino acid amides and peptides according to Günther et al.[199].

Acyl donor ester	Acyl acceptor	Product	Yield (%)
Pbu-OGp	H-Leu-NH_2	Pbu-Leu-NH_2	98
Pbu-OGp	H-Lys-NH_2	Pbu-Lys-NH_2	96
Pbu-OGp	H-Ala-Pro-OH	Pbu-Ala-Pro-OH	93
Pbu-OGp	H-AFAAG-OH	Pbu-AFAAG-OH	92
Bz-β-Ala-OGp	H-Leu-NH_2	Bz-β-Ala-Leu-NH_2	98
Bz-β-Ala-OGp	H-Lys-NH_2	Bz-β-Ala-Lys-NH_2	93
Bz-β-Ala-OGp	H-Ala-Pro-OH	Bz-β-Ala-Ala-Pro-OH	91
Bz-β-Ala-OGp	H-AFAAG-OH	Bz-β-Ala-AFAAG-OH	93

Table 12.5-9. Clostripain-catalyzed coupling of non-amino acid-derived carboxyl and amine components[a] according to Günther and Bordusa[200].

Acyl donor	Acyl acceptor	Product	Yield (%)
Pbu-OGp	H_2N	Pbu-NH	81
Pbu-OGp	H_2N	Pbu-NH	80
Pbu-OGp	H_2N	Pbu-NH	53
Pbu-OGp	H_2N OH	Pbu-NH OH	65
Pbu-OGp	H_2N OH	Pbu-NH OH	78
Pbu-OGp	H_2N OH	Pbu-NH OH	70
Pbu-OGp	H_2N OH OH	Pbu-NH OH OH	92
Pbu-OGp	H_2N NH_2	Pbu-NH NH_2	95
Pbu-OGp	HO	Pbu-O	n.s.
Bz-OGp	H_2N	Bz-NH	82
Bz-OGp	H_2N	Bz-NH	76
Bz-OGp	H_2N	Bz-NH	56
Bz-OGp	H_2N OH	Bz-NH OH	57
Bz-OGp	H_2N OH	Bz-NH OH	84
Bz-OGp	H_2N OH	Bz-NH OH	70
Bz-OGp	H_2N OH OH	Bz-NH OH OH	82
Bz-OGp	H_2N NH_2	Bz-NH NH_2	94
Bz-OGp	HO	Bz-O	n.s.[b]

a Conditions: 0.2 M HEPES-buffer (pH 8.0), 0.1 M NaCl, 0.01 M $CaCl_2$, 5% DMF, 25 °C, (acyl donor): 2 mM, (acyl acceptor): 12 mM; b n.s., no synthesis.

12.5.3.6.3 Anionic Substrate Mimetics

Owing to the general validity of the concept of substrate mimetics Günter and Bordusa[201] have expanded this strategy to anionic leaving groups in the appropriate mimetic structures based on the specificity determinants of Glu-specific endopeptidases. Since the leaving group moiety of a substrate mimetic binds in place of the specificity-determining amino acid side chain, for the strong Glu-preferred V8 protease from *Staphylococcus aurens* a carboxylate function linked with a suitable spacer was chosen as the ester leaving group. Unfortunately, the so far unknown 3D structure of this enzyme allows only the design of suitable mimetic structures by

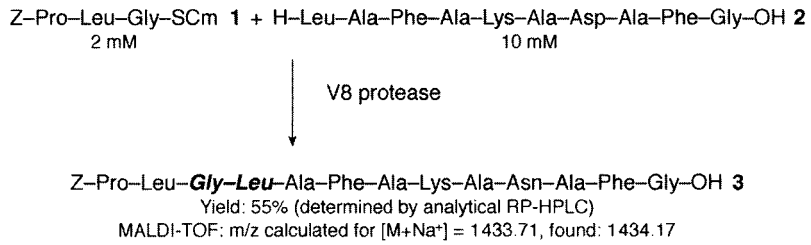

Figure 12.5-20. V8 protease-catalyzed (3 + 10) fragment condensation of Z-Pro-Leu-Gly-Leu-Ala-Phe-Ala-Lys-Ala-Asp-Ala-Phe-Gly-OH[201]. Conditions: 0.2 M HEPES-buffer, pH 8.0, 37 °C, [V8 protease] = 4.9 μM.

empirical structure-function relationship studies. Apart from other structures, the carboxymethyl thioester moiety in particular was selected as a potentially suitable leaving group for imitating the Glu residue in P_1' position. The capability of the carboxymethyl thioester group to act as an artificial recognition site for the V8 protease was initially studied by steady-state hydrolysis kinetic studies. As a general result it can be summarized that the carboxymethyl thioester moiety was found to mediate specific hydrolysis of all carboxymethyl thioester substrate mimetics independently of the P_1' amino acid residue. This also holds for Pro and even for D-Ala, which causes only a slight decrease in specificity compared with the L-enantiomer. Generally, a one to four orders of magnitude lower specificity compared with the common substrate Z-Glu-SMe ($k_{cat}/K_M = 1.12 \times 10^4$ M^{-1} s^{-1}) was found.

In contrast to the non-specificity of the V8 protease for the acyl part, the negative charge of the leaving group is essential to mimick substrates. Lacking this charge in Z-Phe-SCam (-S-CH_2-$CONH_2$ instead -S-CH_2-COOH) a complete loss of specificity results since no hydrolysis of Z-Phe-SCam could be observed. The utility of carboxymethyl thioester for V8 protease-catalyzed peptide synthesis could be demonstrated both by model acyl transfer reactions using amino acid and dipeptides as acyl acceptors and fragment condensation, respectively. Fig. 12.5-20 indicates a semi-preparative (3 + 5) model fragment condensation. After 2 h the enzymatically nonoptimized coupling reaction of **1** with an excess of **2** in HEPES-buffer containing 5% DMSO was stopped by the addition of diluted trifluoroacetic acid and resulted in a yield of 55%.

In addition to carboxymethyl thioesters, Bordusa's group[202] has investigated further types of thioesters and phenylester bearing the carboxyl group, e.g. carboxyethyl thioester, 2-carboxyphenyl thioester, 3- and 4-carboxyphenyl ester, which also mediate acceptance by V8 protease. It is surprising to note that despite the lower degree of structural similarities, the aromatic part of the leaving group led to even higher specificity constants than found for the aliphatic counterparts. In addition, these studies have been expanded to the use of the not so expensive but equally Glu-specific endopeptidase from *Bacillus licheniformis* (BL-GSE), which can easily be purified from alcalase in good yields.

12.5.3.6.4 Hydrophobic Substrate Mimetics

In addition to the enzymes mentioned above with a high specificity for positively and negatively charged P_1' amino acid residues, a third important class of enzymes are represented by peptidases with specificity for aromatic and hydrophobic functionalities. Well-known representatives of this family are the serine peptidases chymotrypsin and subtilisin, which have application in classical enzymatic peptide synthesis and, therefore, they should also be interesting biocatalysts for the substrate mimetic approach. Both enzymes primarly prefer bulky hydrophobic and aromatic P_1' amino acid residues. In addition, the S_1 binding pocket of subtilisin contains a carboxylic acid moiety (Glu^{156}) which causes additional activity towards Arg and Lys[203]. For this reason, aromatic leaving groups with additional positively charged substitutions, e.g. 4-guanidinophenyl ester should fit the natural specificity of these peptidases.

Parallel to an empirical design of specific mimetic structures, the well-known 3D structures of the two enzymes allow the use of rational approaches such as the computer-assisted protein-ligand docking approach. Using the latter to predict the function of the 4-guanidinophenyl ester functionality, Bordusa and co-worker selected Boc-Ala-OGp as a model ligand and docked it towards the enzyme[191]. Fig. 12.5-21 shows the arrangement of the ligand Boc-Ala-OGp at the active site of chymotrypsin in the lowest energy complex (A) in comparison with that found for trypsin (B)[198]. In analogy to the natural specificity of chymotrypsin, hydrophobic contacts between the phenyl moiety of the ester group and the residues Cys^{191} and Val^{213} of the enzyme predominante. Interestingly, the guanidino functionality favors this binding mode by formation of additional hydrogen bonds with three serine residues which are located at the bottom of the S_1 binding pocket. This specific binding pattern, specifically, the orientation of the carbonyl oxygen to Gly^{193}

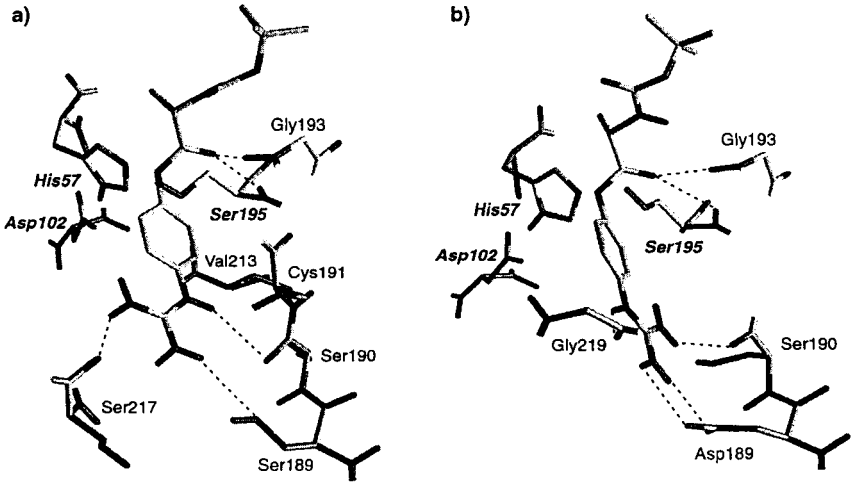

Figure 12.5-21. Arrangements of Boc-Ala-OGp at the active site of chymotrypsin (a) and trypsin (b), respectively according to Günther, Thust, Hofmann and Bordusa (see e.g. reference[191]).

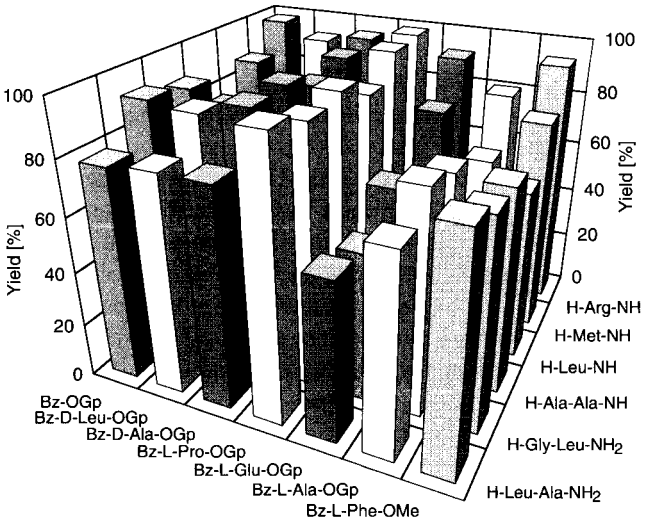

Figure 12.5-22. Chymotrypsin-catalyzed peptide synthesis using 4-guanidinophenyl esters of various non-specific and non-coded acyl moieties [191].

(oxyanion hole), the distance between the carbonyl *C*-atom of the scissile ester bond and the active Ser^{195}, and the reversed binding of the acyl moiety fulfill the conditions for the binding and catalytic mechanism of substrate mimetics. Indeed, acyl 4-guanidinophenyl ester was hydrolyzed by chymotrypsin, and also peptide bond formation using various 4-guanidinophenyl esters with nonspecific coded and non-coded acyl residues could be successfully performed as shown in Fig. 12.5-22 [191]. The yields obtained are in the same range as the yield obtained using the normal-type acyl donor Bz-Phe-OMe.

Furthermore, phenyl ester are also suitable substrate mimetics for chymotrypsin-catalyzed peptide synthesis, as was established by Bordusa's group and will demonstrated by sophisticated fragment condensations in Sect. 12.5.3.7.

12.5.3.6.5 Enzymochemical Substrate Mimetic Approach

In order to synthesize longer polypeptides and proteins the condensation of the initial fragments is an essential prerequisite. Despite different chemical ligation techniques in the field of protein semisynthesis (cf. 12.5.3.1, p. 820–821) enzymatic C - N ligation seems to be the only way to avoid partial epimerization, which cannot be completely eliminated in the course of a chemical fragment coupling reaction. Consequently, the application of the substrate mimetic strategy for the peptidase-mediated condensation of peptide fragments indisputably needs to be combined with the solid-phase peptide synthesis approach. Since a peptide ester can be achieved using of the oxime resin startegy [204, 205] Cerovsky and Bordusa [206] developed a procedure for the synthesis of peptide fragments in the form of substrate mimetics esterified as 4-guanidinophenyl-, phenyl- and mercaptopropionic acid esters. The synthesis protocol involves covalent attachement of the first N^{α}-Boc-protected amino acid to the oxime resin, blocking free hydroxylic groups by acetic

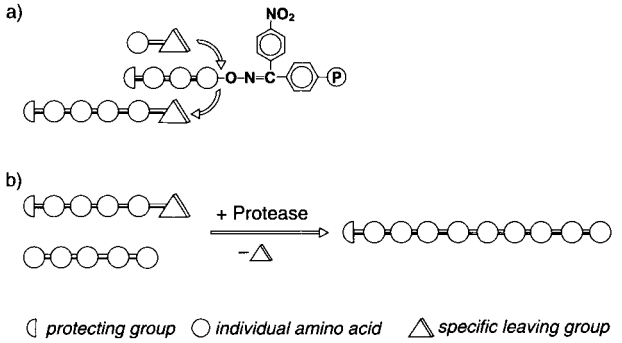

Figure 12.5-23. General approach to fragment substrate mimetics via the oxime resin strategy (a) and substrate mimetic-supported peptide fragment condensation (b) catalyzed by specific peptidases according to Cerovsky and Bordusa[206].

anhydride, deprotection of the N^{α}-amino group of the attached amino acid, followed by successive chain elongation according to the well-known SPPS methodology. The generation of the peptide fragment in the form of the substrate mimetic can be performed by aminolysis of the oxime ester linkage between the peptide and resin, with the appropriate free amino acid substrate mimetic ester as shown schematically in the upper part (a) of Fig. 12.5-23. After deprotection of side-chain functions of the amino acid residues, and if necessary also those of the ester leaving group, the only N^{α}-protected peptide ester can be coupled with an amine component using the suitable peptidase (b). Some examples of model fragment condensations using this approach with catalysis from three different peptidases are given in Fig. 12.5-24. The coupling reactions were performed on a preparative scale using 1 : 2 ratios of acyl donor ester to the nucleophilic acyl acceptors (in the case of trypsin 1 : 2.5) resulting in product yields between 60–70%.

12.5.3.7
Planning and Process Development of Enzymatic Peptide Synthesis

The high enantio- and diastereoselectivity in peptidase-catalyzed peptide synthesis allows, in constrat to most chemical coupling methods, the formation of peptide bonds without partial epimerization in the *C*-terminal amino acid residue of the carboxyl component. Furthermore, owing to the regiospecificity of the enzymes, tedious protection/deprotection steps are not problems in the enzymatic approach. Using serine and cysteine peptidases a further point needs to be decided; namely, should the carboxyl component be used as the acylamino acid or should an ester be used in order to favor acylation of the enzyme. Enzymatic synthesis using peptide esters or amino acid esters as substrates has the clear advantage of proceeding at a high rate, thereby demanding a low concentration of enzyme and, furthermore, being completely independent of the solubility of the starting materials and product. Although the kinetically controlled synthesis would be preferable, the decision should depend on the total synthetic concept. An unfavorable nucleophile specificity may be better taken care of in an equilibrium-controlled reaction with the necessary manipulations of conditions. In spite of some limitations the equilibrium-controlled approach has proved to be worthwhile in the trypsin-catalyzed semisynthesis of

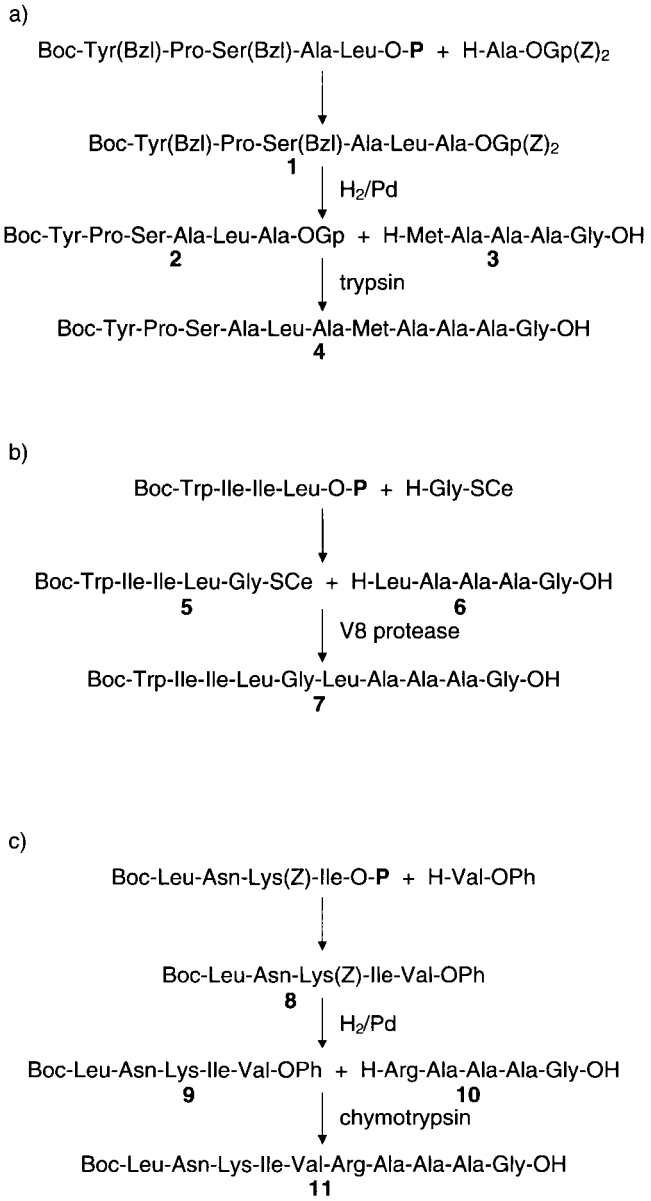

Figure 12.5-24. Combination of solid-phase peptide synthesis and substrate mimetic-supported segment condensations with different peptidases and substrate mimetics according to Cerovskyy and Bordusa[206].

human insulin as well as the industrial aspartame synthesis using thermolysin. In order to overcome poor solubility of the starting components the introduction of solubilizing protecting groups is frequently necessary.

12.5.3.7.1 Stepwise Chain Elongation

Contrary to chemical synthesis, enzymatic stepwise chain building may start either from the *N*-terminus or from the *C*-terminus. In chemical synthesis, incremental chain lengthening from the *N*-terminus, as performed in ribosomal protein synthesis, is normally not recommended under preparative conditions, since the efforts needed to avoid the permanent risk of partial epimerization outweigh the potential gain. Despite these principal limitations, investigations on solid-phase peptide synthesis in an *N*-to-*C* direction, called inverse synthesis, has been performed using HOBt salts of the amino acid 9-fluorenylmethyl esters[207]. Unfortunately, the racemization problem could not be excluded. Furthermore, Mitin and Ryadnov[208] have described inverse peptide synthesis in order to exclude deprotection reactions at every solution synthesis stage. This could be realized using the high solubility of free amino acids in dimethylformamide containing $Ba(ClO_4)_2$, $Ca(ClO_4)_2$ or $Ca(NO_3)_2$. An attempt to solve the extensive exclusion of racemization was tried using copper(II) ions ($CuCl_2$) during activation of the carboxyl group with ethyldimethylaminopropylcarbodiimide (EDC) as the coupling reagent in the presence of HOBt[209].

In a general sense exopeptidases should be the enzymes of choice for stepwise chain assembly since once formed the internal peptide bonds of the growing chain can no longer be proteolytically cleaved from this type of peptidase. Carboxypeptidase exhibit superior properties for the stepwise synthesis, especially, carboxypeptidase Y (CPD-Y)[210] or other serine peptidases of this type. In principle, aminopeptidases can also be used starting from the *C*-terminus. Because under these conditions not only the carboxyl component but also the amine component has a free α-amino function, product isolation is more difficult, particularly, if one component is used in excess. Otherwise, stepwise synthesis from the *C*-terminus is not a problem in chemical peptide synthesis.

A classical example for a kinetically controlled synthesis starting from the *N*-terminus and using CPD-Y as an enzyme for all coupling steps was described by Widmer et al.[211] for [Met]enkephalin (Tyr-Gly-Gly-Phe-Met). Bz-Arg-OEt was coupled with H-Tyr-NH_2 at pH 9.6 giving the Bz-dipeptide amide in 85% yield. The CPD-Y-catalyzed deamidation at pH 9.6 provided Bz-Arg-Tyr-OH in 90% yield. After chemical esterification with EtOH/HCl, the resulting Bz-Arg-Tyr-OEt was coupled with H-Gly-OEt at pH 9 to give the protected tripeptide derivative (yield: 60%), followed by the successive addition of the other amino acid derivatives in the same manner. Amino acid amides were preferred as the amine components, since free amino acids (except Met) only give low yields and amino acid esters give rise to side reactions that are difficult to control.

Finally, the protecting group for the N^{α}-amino function of Tyr, the Bz-Arg moiety, was easily removed with trypsin. The disadvantage of this synthesis strategy seems to be the complicated route of selectively removing the *C*-terminal amide grouping by means of CDP-Y. This step followed by chemical esterification of the peptide had to be resorted to before it was possible to use the intermediate in the next coupling reaction as the carboxyl component.

A second step-by-step peptide synthesis from the *N*- to *C*-terminus was described

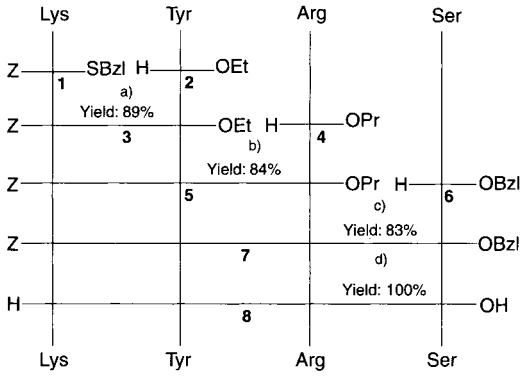

Figure 12.5-25. Synthesis of H-Lys-Tyr-Arg-Ser-OH from N- to C-terminus using clostripain and chymotrypsin, respectively, as biocatalysts according to Bordusa et al.[208]. a) and c): Clostripain; b): chymotrypsin; d): catalytic hydrogenation using 10% Pd/C; -OPr, propyl ester; -SBzl, thiobenzyl ester.

by Bordusa et al.[212] for the model tetrapeptide H-Lys-Tyr-Arg-Ser-OH but using the endopeptidases clostripain and chymotrypsin as biocatalysts (Fig. 12.5-25). The synthesis could be performed without side chain protection for all trifunctional building blocks and the only nonenzymatic reaction was the final catalytic hydrogenation for cleavage the terminal blocking groups.

As a rule, peptidases can only make a meaningful contribution to a synthesis strategy if the full advantagee of the enzymatic reactions can be utilized. An *a priori* completely unrealistic position is the comparison of a stepwise peptidase-catalyzed assembley of a peptide chain with the automatic solid-phase technique.

On the other hand, selected di- and tripeptides can be synthesized enzymatically using solubilizing protecting groups on a large scale, even in a continuous process[118–120] (cf. 12.5.3.4.1, p. 832–833). In addition, the solid-to-solid conversion has proven to be a very useful method for the synthesis of selected short peptides which fulfil the requirements for this special synthetic procedure (cf. 12.5.3.4.2, p. 838–839).

12.5.3.7.2 Fragment Condensation

This approach has some advantages over the stepwise strategy. Firstly, if small fragments are combined to make one which is larger its isolation is more easily facilitated in contrast to a stepwise synthesis, and, secondly, the fragment condensation approach offers the possibility of synthesizing a set of related analoges with variabel sequences in a region. In principle it is possible to synthesize peptides using enzymes both for protection/deprotection procedures as well as for the formation of the peptide bonds. Fig. 12.5-26 shows the fully enzymatic synthesis of the tert.-butyl ester of Leu-enkephalin[213] using both equilibrium and kinetically controlled coupling steps. In order to obtain the unprotected Leu-enkephalin, the *C*-terminal protecting group must be split off by chemical means.

Although, in principal it is possible to perform totally enzymatic synthesis of peptides, in practice combined chemical and enzymatic steps are preferred. For the classical enzymochemical synthesis of polypeptides and even small proteins, the optimum approach is usually synthesis of fragments using the SPPS methodology

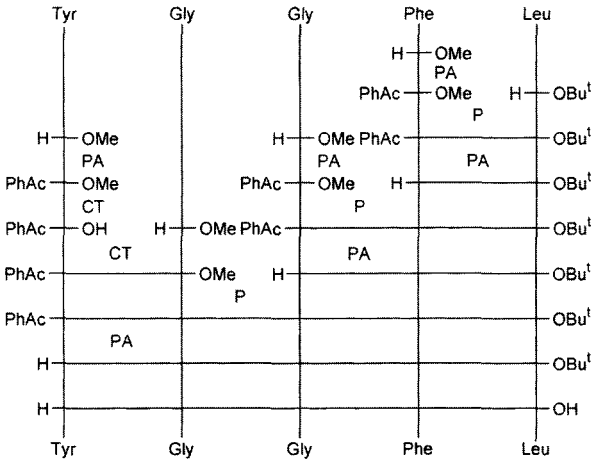

Figure 12.5-26. Fully enzymatic synthesis of [Leu]enkephalin *tert*-butyl ester[209]. PA, penicillin acylase; CT, chymotrypsin; P, papain; PhAc, phenylacetyl.

for enzymatic conjunction in a overall divergent strategy. In a given synthesis project initially it is necessary to separate the whole sequence into segments containing favorable combinations of amino acids which permit peptidase-catalyzed segment coupling according to the eluciated *S'*-subsite specificity. Since the kinetic parameters of the enzymatic synthesis course are often not available, they can be estimated from the data for similar substrates and nucleophilic amine components.

Based on such estimates an optimum synthesis strategy can be established. In Table 12.5-10 selected examples of enzymatically synthesized peptides are compiled. During the last decade in particular, remarkable efforts have been made to find optimum conditions for peptidase-catalyzed peptide synthesis including the development of new reaction conditions and new biocatalyts. Once the optimal synthesis conditions have been recognized, kg amounts of biologically active peptides can be produced. The synthetic biotransformations can normally be achieved with commercially available enzymes which are easy to handle. In addition, owing to the application in only catalytic amounts the higher costs of the enzymes used are usually insignificantly in comparison with highly sophisticated chemical coupling reagents plus the financial expense of the reagents necessary for protection/deprotection procedures in chemical synthesis.

As a model system for peptidase-catalyzed modification of peptides produced by recombinant DNA technology Schellenberger et al.[243, 244] developed a new approach to the production of peptides based on chemical synthesis and peptidase-catalyzed processing (Fig. 12.5-27). First, they produced an artificial substance P precursor as a β-galactosidase (1–459) fusion protein containing nine copies of the sequence *H-Arg-Leu-Arg*-Arg^1-Pro-Lys-Pro-Gln-Gln-Phe^7-OH. The sequence of the peptide precursor was designed to meet the specific requirements of chymotrypsin and papain, respectively, used in conversion reactions as the complete amino acid sequence should be regenerated by addition of the appropriate dipeptide derivatives. After isolation and purification of the fusion protein, which was accumulated in *E. coli* as inclusion bodies, the dodecapeptide ester *H-Arg-Leu-Arg*-Arg^1-Pro-Lys-Pro-

Table 12.5-10. Selected examples of enzymatically synthesized peptides.

Peptide/Protein	Synthesis route*	References
Angiotensin II (analog)	E (part)	[214]
Aspartame	E (total)	[115, 173, 215]
Calcitonin (salmon)	K (part)	[216]
Calcitonin (dicarba analogs)	K (part)	[217]
Caerulein	E (part)	[218]
Caerulein (analog)	E,K (total)	[219]
Cholecystokinin-8	E,K (total)	[219]
	K (part)	[220]
Cholecystokinin-8 (analogs)	K (part)	[221, 222]
Delta sleep inducing peptide (DSIP)	E,K (total)	[223]
Dynorphin-(1–8)	K,E (total)	[224]
EGF (3–14, 21–31, 33–42)	K (part)	[225]
EGF (29–44)	K,E (part)	[226]
Eledoisin (6–11)	E,K (part)	[227, 228]
Eledoisin	K (part)	[229]
[Met/Leu]Enkephalin	E,K (part)	[230]
[Met]Enkephalin	K (total)	[211]
[Leu]Enkephalin	K (total)	[213]
[Leu]Enkephalin derivatives	K (part)	[231]
Growth hormone releasing factor (human) analog	K (part)	[178]
Hepatitis B S antigen (122–137)	K (part)	[232]
Ht31(493–515) peptide	K (part)	[233]
Insulin (human)	E (part)	[116, 117, 145, 234]
Kyotorphin	K (total)	[118, 119, 125]
LH-RH	K (part)	[229, 235, 236]
[D-Phe^6]LH-RH	K(part)	[237]
MSH (5–8, 9–12, 13–16)	K (total)	[238]
Oxytocin (1–9)	K (part)	[239]
Ribonuclease A	K (part)	[161]
Somatostatin	K,E (part)	[240]
Substance P (6–11, 7–11)	K (part)	[227, 241]
Vasopressin (1–6)	K,E (total)	[242]

* E, equilibrium approach; K, kinetic approach; total, totally enzymatic coupling; part, partly enzymatic coupling

Gln-Gln-Phe-Gly^9-OMe was formed by chymotrypsin-catalyzed transpeptidation in the presence of H-Phe-Gly-OMe. In a papain-catalyzed acyl transfer reaction and subsequent tryptic cleavage, the resulting dodecapeptide ester was converted into substance P. These results indicate that peptides can be readily produced by a combination of recombinant DNA technology and peptidase-catalyzed conversion with the advantage of possible incorporation of groups other than coded amino acids into the recombinant product.

The chemoenzymatic synthesis of RNase A [161] using a mutant of subtilisin BNP', called subtiligase, underlines the progress of enzyme-catalyzed fragment condensations in the course of the synthesis of a small protein. The fragments (98–124, 77–97, 64–76, 52–63, 21–51 and 1–20) were synthesized by standard SPPS methodology. The choice of the fragments was solved in such a way that the *C*-terminal residues of the appropriate fragments (Tyr^{97}, Tyr^{76}, Val^{63} and Ala^{20}) were the closest

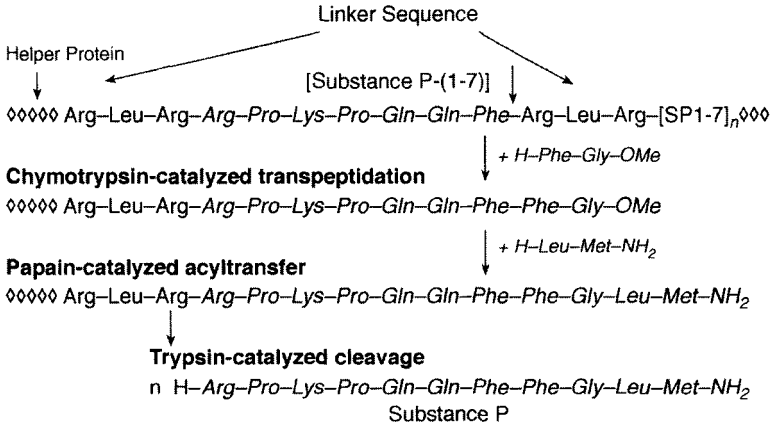

Figure 12.5-27. Peptidase-catalyzed modification of an artificial substance P precursor protein according to Schellenberger *et al.* [243].

to matching the substrate specificity of the subtilisin mutant. Using a considerable excess of the fragments bearing a Phe-NH_2-modified carboxamido methyl ester ensured that most of the side reactions could be suppressed. Starting with the *C*-terminal fragment (98–124) the total yield after five fragment condensations was 15%, and after folding the final protein could be obtained in 8% yield. In a similar manner the three analogs of RNase A were synthesised in which the two residues His^{12} and His^{119} of the active center were exchanged individually and simultaneously for L-4-fluorohistidine.

Despite this impressive example of five successful enzyme-catalyzed fragment condensations with average yields of roughly 75% in the course of the synthesis of RNase A all the peptide bond forming steps could not be performed irreversibly. Even though the new C - N ligation strategy based on the substrate mimetic concept (cf. Sect. 12.5.3.6) has not as yet been proved for the synthesis of a similar protein target, it guarantees the irreversibility of the enzymatic coupling reaction, as can be demonstrated by the chymotrypsin-catalyzed (8 + 16) fragment condensation of the Ht 31(493–515) peptide derived from the human protein kinase A anchoring protein (sequence 493–515) [233]. The synthesis of the 24-peptide was accomplished by the chymotrypsin-catalyzed fragment condensation at a nonspecific Ser-Arg peptide bond via the substrate mimetics strategy (Fig. 12.5-28). The fully protected carboxyl component **1** was synthesized on Kaiser's oxime resin and was released from the support by aminolysis with H-Ser(Bzl)-OP according to the procedure described on p. 850–851. After side-chain deprotection by catalytic hydrogenation, **2** was coupled with the unprotected amine segment **3** catalyzed by chymotrypsin, leading to the complete conversion of both peptide segments. Finally, the *N*-terminal Boc group was cleaved by TFA giving the desired Ht 31 (493–515) peptide **5**.

Boc–Asp(OBzl)–Leu–Ile–Glu–(OBzl)–Glu(OBzl)–Ala–Ala–O–oxime resin

↓ TFA.Ser(Bzl)-OPh

Boc–Asp(OBzl)–Leu–Ile–Glu–(OBzl)–Glu(OBzl)–Ala–Ala–Ser(Bzl)–OPh **1**

↓ H_2/Pd

Boc–Asp–Leu–Ile–Glu–Glu–Ala–Ala–**Ser**–OPh **2**

H–**Arg**–Ile–Val–Asp–Ala–Val–Ile–Glu–Gln–Val–Lys–Ala–Ala–Gly–Ala–Tyr–OH **3**

↓ Chymotrypsin

Boc–Asp–Leu–Ile–Glu–Glu–Ala–Ala–**Ser–Arg**–Ile–Val–Asp– **4**
–Ala–Val–Ile–Glu–Gln–Val–Lys–Ala–Ala–Gly–Ala–Tyr–OH

↓ TFA

H–Asp–Leu–Ile–Glu–Glu–Ala–Ala–**Ser–Arg**–Ile–Val–Asp– **5**
–Ala–Val–Ile–Glu–Gln–Val–Lys–Ala–Ala–Gly–Ala–Tyr–OH

Figure 12.5-28. Chymotrypsin-catalyzed (8+16) segment synthesis of the Ht 31(493–515) peptide via substrate mimetic strategy[233].

12.5.4
Conclusion and Outlook

Despite the fact that chemical methods are popular for the synthesis of peptides a huge number of papers has been published in recent decades dealing both with enzymatic formation of peptide bonds and enzymatic manipulation of protecting groups. Enzymatic methods have several advantages over chemical procedures but at present more peptides are synthesized by chemical synthesis than in peptidase-catalyzed processes. The use of peptide synthesizers, in addition to recent new developments in the field of chemical ligation procedures, still favor chemical methods compared with the enzymatic approach. However, there is no doubt that enzymatic methods have advantages, including the prevention of racemization, no need for time-consuming and expensive protection/deprotection procedures of side-chain functions, the reduced use of problematic (toxic) solvents and reagents and possible reuse of the biocatalysts. The question should not be whether to use a chemical or an enzymatic approach in peptide synthesis; an ingenious combination of chemical and enzymatic steps should promote the general progress in peptide synthesis.

It could be demonstrated that after establishing the optimal synthesis conditions, kg amounts of biologically active peptides and analogs can be obtained using enzymatic coupling methods. The semisynthetic synthesis of human insulin and the

production of aspartame in a ton-scale underline the industrial importance of the enzymatic approach. However, the enzymatic approach does not have the versatility of chemical synthesis methods and suffers from some limitations. The main reason seems to be the lack of a universal enzyme which is capable of catalyzing peptide bond formation for all possible combinations of the 21 proteinogenic amino acid residues located both as *C*- and *N*-terminal building blocks in peptide fragments to be coupled. Such an enzyme could not be developed during evolution due to the extremely high specificity requirements. In ribosomal protein synthesis nature prefers the stepwise synthesis from *N*- to *C*-terminus followed by maturation procedures based on limited proteolysis and further modifications. The only biocatalyst involved in ribosomal synthesis, the peptidyl transferase, seems to be an old ribozyme without any specificity for the P_1 side chain functions of the amino acids, only catalyzing the acyl transfer reaction of the selected aminoacyl-tRNAs. Since such a biocatalyst has no practical importance in peptide synthesis in a peptide laboratory, the only alternative for this purpose is the reverse catalytic hydrolysis potential of proteases.

The advantages and drawbacks of peptidases used for catalyzing peptide bond formation have been demonstrated in this contribution. An ingenious combination of chemical and enzymatic strategies as demonstrated in a new synthesis of RNase A should be the state-of-the-art in this field at present. Furthermore, using the new C - N ligation strategy based on the substrate mimetic concept, irreversible peptide bond formations catalyzed by high specific peptidases can be performed for the first time. In combination with peptidase mutants which lack amidase activity, this new C - N ligation approach will contribute to significant progress in enzymatic peptide synthesis, especially in clear-cut fragment condensations using recombinant polypeptide thioester as the substrate mimetics with chemically synthesized or recombinant fragments. This specific programming of enzyme specificity by molecular mimicry corresponds in practice to a conversion of a peptidase into a C - N ligase, a biocatalyst which could not developed by nature during evolution.

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12.6
Addition of Amines to C = C Bonds

Marcel Wubbolts

The ammonia lyases (E. C. 4.3.1.x), which catalyze the addition of amines to carbon-carbon double bonds, belong to the class of carbon-nitrogen lyases. The reactions catalyzed by ammonia lyases are in equilibrium and depending on reaction conditions the reaction can be directed either towards ammonia addition or in the direction of elimination of ammonia.

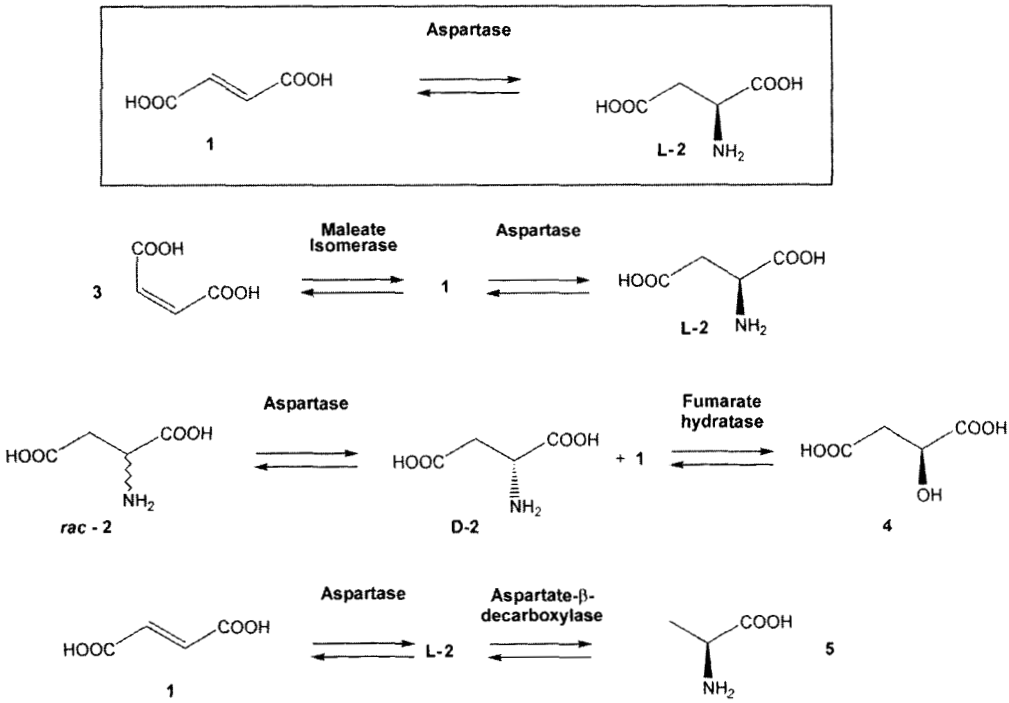
Ammonia lyases in their natural role are involved in the metabolism of amino acids and also play a role in, for instance, the degradation of amino sugars, but only a limited amount of these enzymes have been characterized biochemically. Application of a broad range of different ammonia and lyases in organic chemical synthesis on an industrial scale has thus far not occurred, which is due to both their limited commercial availability and their lack of stability under process conditions. Exceptions are the commercially applied aspartase, which is an ammonia lyase that is utilized for the synthesis of L-aspartic acid from fumaric acid, and phenylalanine lyase. The latter is an example of a commercial application of an ammonia lyase in a process for the production of L-phenylalanine and more importantly L-phenylalanine derivatives.

12.6.1
Addition of Ammonia to Produce Amino Acids

12.6.1.1
Aspartic Acid

L-aspartic acid ammonia lyase, or aspartase (E. C. 4.3.1.1) is used on a commercial scale by Kyowa Hakko, Mitsubishi, Tanabe and DSM to produce L-aspartic acid, which is used as a building block for the sweetener Aspartame, as a general acidulant and as a chiral building block for synthesis of active ingrediants[1]. The reaction is performed with enzyme preparations from *E. coli, Brevibacterium flavum* or other coryneform bacteria either as permeabilized whole cells or as isolated, immobilized enzymes. The process is carried out under an excess of ammonia to drive the reaction equilibrium from fumaric acid (**1**) in the direction of L-aspartic acid (L-**2**) (see Scheme 12.6-1) and results in a product of excellent quality (over 99.9 % e. e.) at a yield of practically 100 %. The process is carried out on a multi-thousand ton scale by the diverse producers of L-aspartic acid. Site directed mutagenesis of aspartase from *E. coli* by introduction of a $Cys_{430}Trp$ mutation has resulted in significant activation and stabilization of the enzyme[2].

Since maleic acid is a cheaper starting material than fumaric acid, the process that is probably the most economical makes use of both a maleate isomerase (E. C. 5.2.1.1) and aspartase (E. C. 4.3.1.1), Scheme 12.6-1. Mitsubishi has succeeded in



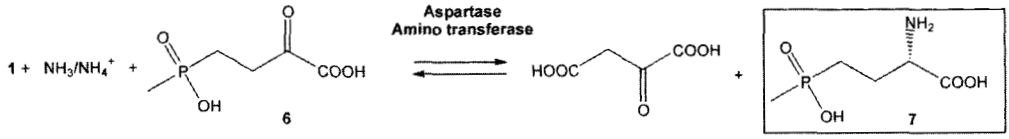
Scheme 12.6-1.

combining both activities in a *Brevibacterium flavum* recombinant for the large-scale production of L-aspartic acid[3].

Mitsubishi has also developed a process for production of D-aspartic acid (D-**2**) and L-malic acid (**4**) by incubation of racemic aspartic acid with the exclusively L-selective aspartase in combination with fumarase, thereby preventing the reaction going backwards by conversion of the generated fumaric acid into L-malic acid[4].

The combined utilization in a single reactor of both aspartase from *Brevibacterium flavum* and aspartate-β-decarboxylase from *Pseudomonas dacunhae*, thereby catalyzing the reaction from fumaric acid via L-aspartic acid to L-alanine (**5**), has also been developed by Mitsubishi[5].

Another combination reaction is the biocatalytic production of the herbicide phosphinotricin [L-2-amino-4-(hydroxymethylphosphinyl)butyric acid, (**7**) in Scheme 12.6-2] by the company Meiji Seika, whereby an amino-transferase that acts on 4-(hydroxymethylphosphinyl)-2-oxo-butyric acid and that utilizes aspartic acid as the amino donor was used in combination with aspartase to generate the amino donor from fumaric acid and ammonia[6].

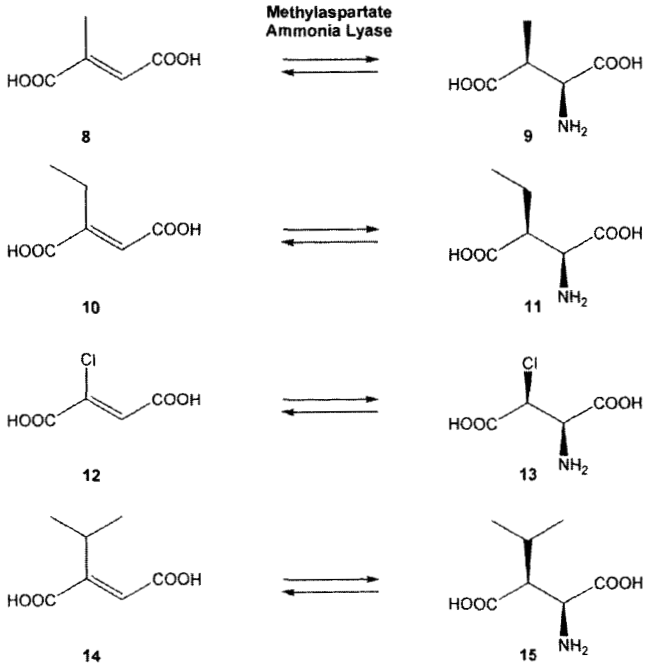


Scheme 12.6-2.

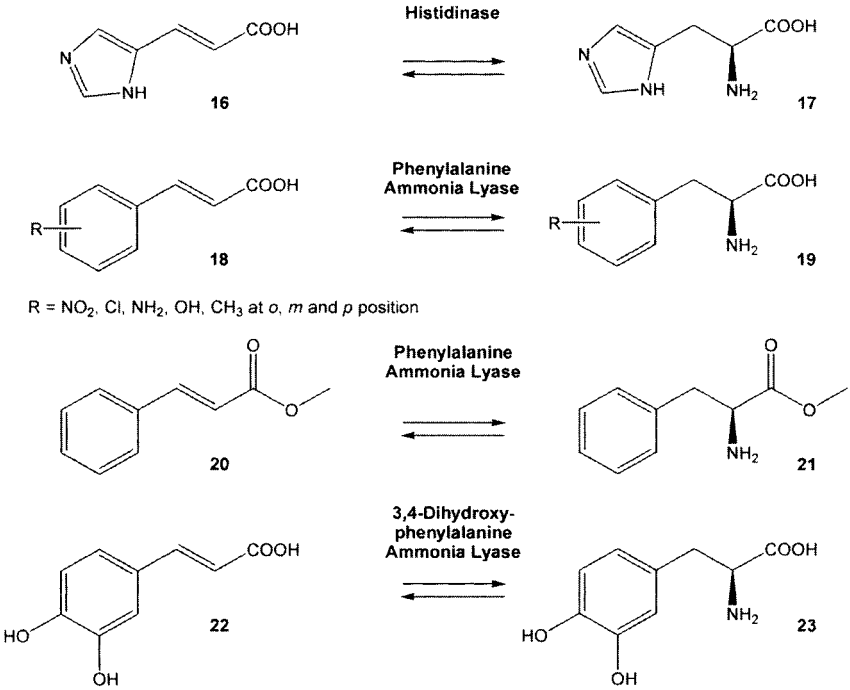
12.6.1.2
Aspartic Acid Derivatives

The enzyme methylaspartate ammonia lyase (β-methylaspartase, E.C. 4.3.1.2) is involved in the metabolism of branched pentanoic acids. The enzyme catalyzes the addition of ammonia to mesaconic acid (**8**) to yield L-*threo*-3-methylaspartate (**9**) as depicted in Scheme 12.6-3. The enzyme has been shown to be induced under anaerobic conditions in facultative anaerobes such as *Citrobacter, Proteus, Escherichia coli* and *Enterobacter*[7, 8] and has been applied for the synthesis of 3-substituted (*S*)-aspartic acid derivatives, such as (*2S,3S*)-3-methylaspartic acid (**9**), (*2S,3S*)-3-ethylaspartic acid (**11**), and (*2R,3S*)-3-chloroaspartic acid (**13**)[7]. In addition, a process for the preparation of dialkyl-(*2S,3S*)-3-ethylaspartates using methylaspartate ammonia lyase has been developed by Merck[9].

Bear et al. have been using methylaspartate ammonia-lyase from *Clostridium tetanomorphum* to produce optically active pure precursors [3-methyl-, 3-ethyl and



Scheme 12.6-3.



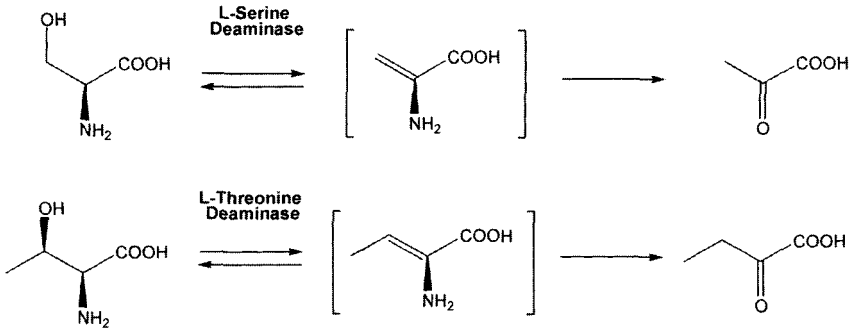
Scheme 12.6-4.

3-iso-propylaspartic acids, (**15**)] for the synthesis of benzyl 3-alkylmalolactonates, which are suitable building blocks for semi-crystalline polyesters[10].

12.6.1.3

Histidine Ammonia Lyase

Histidine ammonia lyase (HAL, histidinase, histidine-α-deaminase, E.C. 4.3.1.3) is capable of abstracting ammonia from L-histidine (**17**), resulting in the formation of urocanoic acid [Scheme 12.6-4, (**6**)], an intermediate in the metabolism of L-histidine[11]. HAL has also been identified as a key enzyme in the synthesis of secondary metabolites such as Nikkomycin in *Streptomyces tendae*[12]. The mechanism of the enzyme has been investigated and seems to proceed via the carbanion intermediate[11, 13]. Synthetic applications of HAL are difficult to achieve, particularly as the enzyme is sensitive to oxygen[13]. The utility of HAL is limited to niche applications such as the synthesis of radiolabeled urocanic acids as tracers of histidine metabolism[11].



Scheme 12.6-5.

12.6.1.4
Phenylalanine, Tyrosin and L-DOPA

Phenylalanine ammonia lyase (PAL, E.C. 4.3.1.5) is an enzyme of relaxed substrate specificity that accepts both *trans*-cinnamic acid (Scheme 12.6-4: (**18**), R = H) and *p*-coumaric acid [(**19**), R = OH] as substrates and thus results in the formation of the natural amino acids L-phenylalanine and L-tyrosine. The enzyme plays an important role in the synthesis of alkaloids, flavenoids and lignin in plants. The reaction has been exploited by Mitsui[14, 15], Great Lakes/NSC[16] and others to implement synthetic routes for non-natural substituted derivatives of L-phenylalanine starting from *trans*-cinnamic acids, for instance using the PAL enzymes from *Rhodotorula rubra, Rhodotorula glutinis* or *Rhodosporidium toruloides*. The PAL mediated synthesis of a variety of L-phenylalanine derivatives, carrying aromatic ring substituents such as nitro-, chloro-, amino-, hydroxy- and methyl groups at the 2, 3 and 4 position have thus been described[16–18]. Also, the synthesis of *N*-heterocyclic molecules, derived from phenylalanine by PAL has been shown[17, 19, 20]. The direct synthesis of phenylalanine methyl ester [Scheme 12.6-4, (**21**)] as a building block for aspartame, from *trans*-cinnamyl methyl ester (**20**) by PAL from *Rhodotorula glutinis* further illustrates the synthetic versatility of PAL[21, 22]. Radioactive tracers derived from L-phenylalanine have also been made with the aid of PAL[23, 24].

An enzyme that is related to PAL, dihydroxy-L-phenylalanine ammonia lyase (E.C. 4.3.1.11), is capable of synthesizing L-DOPA (**23**) from 3,4-dihydroxy-*trans*-caffeic acid (**22**), but this starting material is not as readily available as catechol, pyruvate, and ammonia are. As a result, the tyrosine phenol-lyase (TPL, E.C. 4.1.99.2) of *Erwinia herbicola* is the enzyme of choice for biocatalytic L-DOPA production[25, 26], particularly as productivity has been increased since the TPL encoding gene from *Erwinia herbicola* was cloned and has been overexpressed successfully[25].

12.6.1.5
Serine and Threonine Deaminases

Both the L- and D-serine deaminase catalyze the elimination of the amino functionality of both L- and D-serine, but the mechanism proceeds via the initial elimination of water and these enzymes are thus classified as hydrolyases (L- and D-serine dehydratases E.C. 4.2.1.13 and E.C. 4.2.1.14, respectively)[27, 28]. The aminoacrylate generated is unstable and subsequent elimination of the amine results in the formation of pyruvate. Similarly, threonine deaminase is in effect a dehydratase that converts L-threonine into 2-oxobuturate, water and ammonia (E.C. 4.2.1.16) (Scheme 12.6-1).

12.6.1.6
Ornithine Cyclodeaminase

Ornithine cyclodeaminase (E.C. 4.3.1.12) is an ammonia lyase that is not ubiquitously present but which has been identified in genera such as *Rhizobium, Agrobacterium, Pseudomonas, Rhodobacter* and *Clostridium*[29–32]. Ornithine cyclodeaminase, which contains NAD that is tightly bound to the enzyme, catalyzes the conversion of L-ornithine, an intermediate in the metabolism of L-arginine, into L-proline. The reaction is peculiar among the ammonia lyases in that it involves a deamination of the amino group at the α-position followed by attack of the δ-amino group to give 2-oxo-5-aminopentanoic acid to form L-proline[33]. Conversions other than that from L-ornithine to L-proline have not been described.

12.6.2
Ammonia Lyases that Act on Other Amines

12.6.2.1
Elimination of Ammonia from Ethanolamine

The elimination of ammonia from ethanolamine to give acetaldehyde, which involves vitamin B_{12} and which has been demonstrated to proceed via a radical anion[34, 35], is catalyzed by ethanolamine ammonia lyase (EAL, E.C. 4.3.1.7). Genetic and biochemical analysis of the ethanolamine ammonia lyase isolated from *Salmonella typhimurium* and *Rhodococcus sp.* have been carried out[36, 37] and the enzyme appears to belong to a class of B_{12} dependent enzymes that catalyze similar rearrangements, such as diol dehydratase and methylmalonyl-CoA mutase[35]. Ethanolamine ammonia lyases are induced under anaerobic conditions, which is required since the radical reaction intermediates are highly reactive with dioxygen[38]. Despite the interesting chemistry, we did not come across synthetic applications of ethanolamine ammonia lyases, other than the observation that the enzyme of *Acetobacterium* catalyzes the elimination of ammonia from triethanolamine in addition to ethanolamine[38].

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12.7 Transaminations

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12.7.1 Introduction

Given their critical role in biological systems, it is not surprising that numerous applications for amino acids have developed, particularly in the pharmaceutical industry. Fourteen of the twenty common proteinogenic L-amino acids are essential in human diets, which has led to the development of a significant market for these as components in intravenous feeding solutions. L-Glutamic acid is used as a flavor enhancer in foods with annual sales estimated at greater than one billion dollars. L-Lysine, D,L-methionine, and L-threonine have already become established as large-volume additives to animal feeds that require enrichment in certain deficient amino acids, and L-tryptophan is developing a similar application. L-Phenylalanine and L-aspartic acid have very important markets as key components in the manufacture of the high-intensity sweetener aspartame. A competitive product in development, alitame, is synthesized from D-alanine.

The importance of non-naturally occurring amino acids can be seen from the increasing number of pharmaceutical products that incorporate one or more such compounds as intermediates. Numerous chiral drug candidates are synthesized from various natural and non-natural amino acid building blocks and have been submitted for biological testing. Inevitably, applications for amino acids, both naturally-occuring and non-natural, will result from this activity. There are already numerous examples. The synthesis of two thrombin inhibitors, Tirofiban from Merck & Co. and Inogatran from Astra-Zeneca, is based on analogs of L-tyrosine and D-cyclohexylalanine, respectively. D-2-Aminoadipic acid is one of the amino acids found in the tripeptide that is converted biologically into the β-lactam nucleus, and its use as a precursor for producing semi-synthetic penicillins and cephalosporins has been suggested. The L-antipode is also a common component of combinatorial synthesis approaches that incorporate non-naturally occurring amino acids. Fluorine substitution is also becoming increasingly common in the preparation of peptide analogs. In particular, *p*-fluoro-L-phenylalanine is a good choice as a non-naturally occurring amino acid for such work because it is almost isosteric with L-phenylalanine, but contains a strongly electron withdrawing fluorine atom to modify its dipole moment.

In particular, the non-naturally occurring amino acid L-*tert*-leucine has received significant attention due to several pharmaceutically active compounds into which it is incorporated[1]. HIV-protease inhibitors developed by Novartis and Abbott are based on L-*tert*-leucine[2, 3]. Roche has developed the anti-arthritic compound Ro 31–9790 based on its potent inhibition of collagenase[4] and a key component in the synthesis of Ro 31–9790 is the methylamide of L-*tert*-leucine. Boehringer Ingelheim developed a series of compounds that inhibit the ribonucleotide reductase of Herpes

simplex virus; several of the most active structures contained L-*tert*-leucine[5]. As a result, a market for L-*tert*-leucine as a pharmaceutical intermediate is developing. In addition, the derivative L-*tert*-leucinol is widely used as a chiral auxiliary[6].

The markets for non-naturally occurring amino acids can be substantial. Inhibitors of angiotensin-converting enzyme, or the so-called ACE inhibitors, have developed strong markets as anti-hypertensive drugs. One of the most successful is the product Enalapril, which has achieved sales of more than $1 billion annually. Marketed by Merck, Sharpe and Dohme, Enalapril is due to go off patent soon, leading to the emergence of generic competitors. Other similar ACE inhibitors include Ramipril, Benazapril, Lisinopril, Zestril, Trandolopril, and Quinipril. A key component in all of these compounds is L-4-phenyl-2-amino-*n*-butanoic acid, or L-homophenylalanine. As price competition for generic ACE inhibitors intensifies, L-homophenylalanine will probably become an important non-naturally occurring amino acid product. Other large volume products include D-phenylglycine and D-*p*-hydroxyphenylglycine, key intermediates in the synthesis of ampicillin and amoxicillin, respectively, D-penicillamine, a chelator used to treat cystinuria and severe arthritis, D-valine, a building block for the synthetic pyrethroid Fenvalerate, and phosphinothricin, an important herbicide marketed by AgrEvo. Additional commercial opportunities exist for the production of isotopically labeled amino acids, particularly 15N, 15N/13C, and 15N/13C/2H amino acids for use in medical research, with a larger potential market in magnetic resonance imaging.

Various methods have been developed for the production of amino acids. Most naturally-occurring, proteinogenic amino acids can be produced by fermentation, although chemical synthesis, isolation from hydrolyzed proteins, and enzymatic conversion are used in a few instances. For the production of non-proteinogenic or non-natural amino acids for which no metabolic pathways exist, traditional fermentation methods cannot be used without re-engineering of the metabolic pathways in the cell. For these types of amino acids, various chemical and enzymatic synthetic methods have become increasingly common.

Among the various enzymes capable of producing optically-active amino acids, transamination reactions, catalyzed by enzymes known as aminotransferases or transaminases, have broad potential for the synthesis of a wide variety of enantiomerically pure (*R*)- and (*S*)-compounds containing amine groups. Indeed, various examples of the use of aminotransferases for the production of D- and L-amino acids, both naturally-occurring and non-natural, have been published[7–15]. In addition, certain aminotransferases have been found to act on amines, and methods for the production of enantiomerically pure amines by transamination have been described[16–21]. This method allows for yields of up to 100% whereas routes based on hydrolases require external racemization to reach such yield levels. In this section we will focus on the application of aminotransferases.

12.7.2
Description of Transaminases

12.7.2.1
Homology and Evolutionary Subgroups of Aminotransferases

About one third of all known sequences of vitamin B6-dependent enzymes belong to aminotransferases which in turn can be divided into four subgroups based on sequence homology: the most common species such as aspartate, tyrosine, or phenylalanine aminotransferase belong to subgroup I, subgroup II takes (acetyl)ornithine, ω-amino acid and γ-aminobutyrate aminotransferases, subgroup III comprises the D-amino acid transferases, and subgroup IV the (phospho)serine aminotransferases[22]. Only 4 of the about 400 amino acid residues proved to be invariant among all aminotransferase sequences: Gly 197, Asp/Glu 222, Lys 258, and Arg 386. Apparently, aminotransferases form a group of homologous proteins, the chemistry of which already existed very early in evolution.

12.7.2.2
Mechanism of Transamination

Aminotransferases are key enzymes in a number of metabolic pathways, and as a result, enzymes from this class are widely distributed in nature. The first evidence for the presence of an enzyme catalyzing a transamination reaction was published by Needham and Szent-Györgyi and co-workers who noticed a relationship between the L-glutamic acid, L-aspartic acid, and oxaloacetic acid levels in pigeon breast muscle[23]. Banga and Szent-Györgyi demonstrated the reversibility of glutamic-pyruvic transaminase (E.C. 2.6.1.2, alanine aminotransferase) by chemically isolating the amino acid products L-glutamate and L-alanine[24, 25]. Since that time, a large number of different aminotransferases have been discovered and characterized, including aminotransferases, capable of catalyzing the transamination of all naturally-occurring amino acids. There are now more than 2500 sequences of aminotransferases known, compared with 51 sequences in 1993[22]. As of the middle of February 2001, the Entrez databank contained 121 3D-structures of 9 aminotransferases from 13 organisms.

The mechanism of the reaction is well understood as a result of the detailed studies of Meister[26, 27]. Aminotransferases catalyze the transfer or an amino group from an amino acid donor to a 2-ketoacid acceptor (Fig. 12.7-1). This amino group transfer is mediated by the cofactor pyridoxal phosphate, which is reversibly bound to the enzyme through a Schiff-base linkage to the epsilon-amino group of an active-site lysine. Mechanistically, the reaction catalyzed by an aminotransferase can be thought of as the result of two discrete steps. The first step is the transfer of an amino group from the amino group donor to pyridoxal phosphate, generating a 2-ketoacid byproduct that dissociates from the enzyme and an enzyme-bound pyridoxamine phosphate intermediate. The second step involves the transfer of the amino group from the enzyme-bound pyridoxamine phosphate to the 2-ketoacid acceptor, and

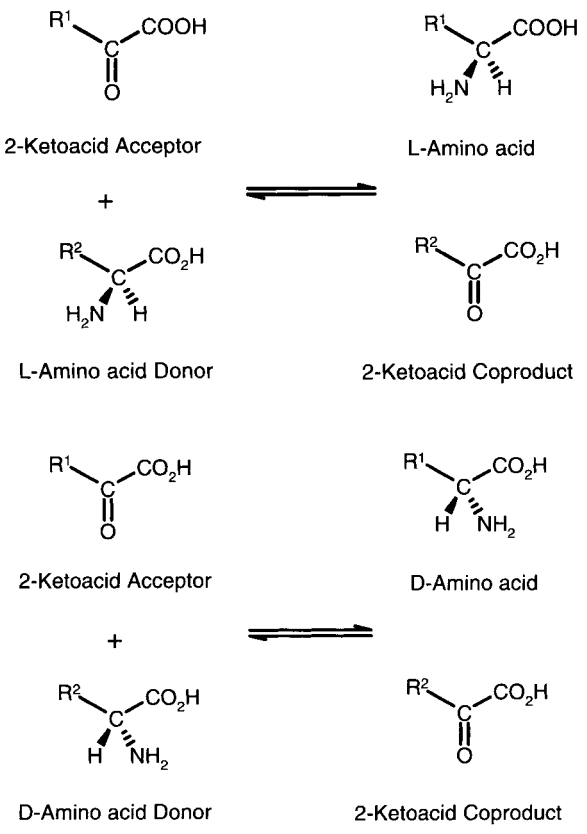

Figure 12.7-1. General reaction catalyzed by aminotransferases.

producing the corresponding amino acid product and regenerating the pyridoxal phosphate cofactor for another catalytic cycle. As a result, aminotransferases characteristically exhibit ping-pong kinetics [28].

12.7.2.3
Protein Engineering and Directed Evolution with Aminotransferases

Aminotransferase (AAT), the enzyme catalyzing the reversible transformation of aspartate and glutamate into the respective oxo acids, has been studied most among the vitamin B6-dependent enzymes. An X-ray crystal structure is now known for the aspartic-glutamic aminotransferase from *E. coli* [29]. Active site residues have been identified, laying the groundwork for further detailed mechanistic studies and modification of the enzyme by specific mutagenesis. Several workers have been successful at changing the relative activity of aminotransferase towards different groups of substrates or even different reactions through structure-based protein engineering and directed evolution.

12.7.2.3.1 Structure-based Protein Engineering

Multiple active-site site-specific mutations of AAT led to an increase in β-decarboxylase activity with the double mutant Y225R/R386A (1380-fold)[30]. Coupled with a decreased transaminase activity by a factor of 500 in the single mutant R292K[31], workers found a combined 20 000-fold decrease in the rate of transamination in the triple mutant Y225R/R292K/R386A[32]. In fact, the triple mutant catalyzed β-decarboxylation 8-fold faster than transamination, a change of ratio from the wild-type enzyme by a factor of 25 million. The observed changes in substrate specificity were rarely additive however, because triple mutants containing R292X, i.e. mutations to amino acids other than lysine, were mostly completely inactive towards β-decarboxylation even though they contained the double mutant Y225R/R386A eliciting β-decarboxylase activity.

Previously, AAT had been transformed into an L-tyrosine aminotransferase (TAT) by site-specific mutation of up to six amino acid residues lining the active site of wild-type TAT. The hexuple AAT-mutant achieved kinetic data towards the transamination of aromatic substrates such as L-phenylalanine within an order of magnitude of wild-type TAT[33].

12.7.2.3.2 Directed Evolution of Aminotransferases

Meanwhile, directed evolution methods that combine mutagenesis of genes with high-throughput screening of functional gene products have developed rapidly.

In a selection strategy based on the substrate 1-phenyl-*n*-propylamine (PPA) as the sole source of nitrogen in a chemostat, a recombinant *Pseudomonas putida* strain carrying the *R*-transaminase gene, a single amino acid change, Y112F, presumably at or near the active site, improved enantioselectivity of the reaction of racemic 1-phenyl-*n*-propylamine to (*S*)-1-phenyl-*n*-propylamine and propiophenone to 37.8% e.e. from 6.5% e.e. in the wildtype[19]. Further site-directed mutagenesis of position 112 yielded 99.4% e.e. in the mutant Y112L. In a related example, a single mutant T51S, generated by error-prone PCR in about 10000 samples, both improved tolerance of (*R*)-transaminase towards the reaction product, a substituted 1-phenyl-2-propylamine (an amphetamine), from 85 to 105 mM as well as reaction rate[19]. Lastly, a β-tetralone was converted into the corresponding (*S*)-amine in 65% e.e. by wild-type (*S*)-transaminase (Fig. 12.7-2)[19]. Random mutation followed by activity screening for the colored ketone starting from the enantiomerically pure amine, produced a number of single mutants such as M245V, P247L, and F407L with higher enantioselectivity, up to 84% e.e., at similar level of activity. It was further found that combination of advantageous mutants through site-directed mutagenesis around

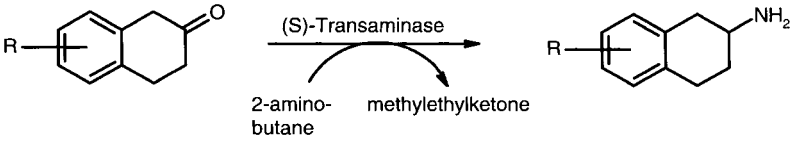

Figure 12.7-2. Conversion of tetralone-2 to 2-aminotetraline by (*S*)-transaminase.

sensitive sites such as 245–247 and 405–407 improved enantioselectivity further, up to 94%.

Efforts to evolve aminotransferases with improved activity on new ketoacid substrates have been initiated with encouraging results [34]. Using directed evolution, the substrate specificity of AAT has been changed to one favoring β-branched amino acids and their respective oxoacids, effectively converting AAT into a branched-chain aminotransferase (BCAT). By employing an *E. coli* auxotroph deficient in the branched-chain aminotransferase (BCAT) gene, *ilvE*, the authors set up a stringent selection system which provided a powerful advantage for cell growth to the mutated AAT systems [35, 36]. The resulting evolved aminotransferases had 13 [35] and 17 [36] amino acid substitutions and showed 10^5-fold and 2×10^6-fold improvement in catalytic efficiency (k_{cat}/K_M), respectively, towards the unnatural substrate, valine, and between 10- and 100-fold decrease towards the natural substrate, L-aspartate, compared with the wild-type. A high degree of conserved amino acid substitutions was found in most active mutants. Interestingly, only one mutated amino acid residue in each case is located at a distance to the substrate that would allow interactions, the remainder were mutated far away from the active site. This work demonstrates that 10^6-fold shifts in substrate specificity can be achieved when employing directed evolution methods, that combinatorial or evolutionary methods are probably superior to rational design methods when changing substrate specificity, and most importantly, that remote residues and their interactions with the active site environment are important determinants of enzyme activity and specificity. Such remote residues act cumulatively, possibly by remodelling the active site, by altering the subunit interfaces, or by shifting different enzyme domains.

12.7.3
Use of Aminotransferases in Biocatalytic Reactions

12.7.3.1
Synthesis of α-L-Amino Acids

Aminotransferases (transaminases) have been studied as potentially useful biocatalysts for the production of a wide range of different amino acids. The general reaction catalyzed by aminotransferases is shown in Fig. 12.7-1. An amino group is transferred from a donor amino acid to a 2-keto acid acceptor. As described earlier, a cofactor, most commonly pyridoxal phosphate, is involved in the catalysis. The cofactor, which is only required in concentrations of 50–100 μM, is reversibly bound to the enzyme through a Schiff-base linkage to the epsilon-amino group of active-site lysine [26–28]. Using an aminotransferase, a desired amino acid can be produced from a given 2-keto acid precursor using an inexpensive L-amino acid as the amino group donor. As a co-product of the reaction, a second 2-keto acid corresponding to the amino acid donor is produced along with the desired amino acid product in equimolar amounts.

Among the advantages of transaminases as biocatalysts for the production of optically pure amino acids are as follows:

- Aminotransferases have high stereoselectivity for a given enantiomer. Optically active L- or D-amino acids are produced stereoselectively; the process is a chiral synthesis, not a resolution.
- The catalytic rates of these enzyme-catalyzed reactions are generally relatively rapid.
- Capital costs for such a biocatalytic process are low; in contrast to the situation with fermentations, already existing chemical process equipment can be used for performing the enzyme-catalyzed reaction.
- A large number of the required 2-keto acid precursors are accessible through chemical synthesis, expanding the range of potential products.
- Aminotransferases are potentially applicable to the production of a wide range of amino acids, because enzymes are available for D- and L-amino acids. In addition, a wide range of aminotransferases with side-chain specificity are known, including enzymes for the production of amino acids with aromatic side chains, acidic side chains, branched alkyl side chains, etc.
- In some cases, the 2-keto acid by-products may also have significant value. For example, important markets exist for pyruvic acid, 2-ketoglutaric acid, and other similar compounds.

One of the simplest examples of an efficient transamination process is the production of L-alanine and 2-ketoglutarate from the precursors L-glutamate and pyruvic acid (Fig. 12.7-3). Porcine glutamic-pyruvic transaminase is available commercially, and this enzyme was used as a model system for studying the transamination on a preparative scale. The equilibrium constant was measured for this reaction and found to be 1.86, slightly favoring the formation of L-alanine and 2-ketoglutarate. Glutamic-pyruvic transaminase was immobilized on porous aminopropyl glass using water-soluble carbodiimide as a coupling agent[7]. At a loading of 20 mg of total protein bound per gram of glass, the activity of the biocatalyst when assayed or the production of L-alanine was 400 units per gram of biocatalyst. The enzymatic activity retained after immobilization was 40%, and the immobilized enzyme was used for the continuous production of L-alanine and 2-ketoglutarate from pyruvate

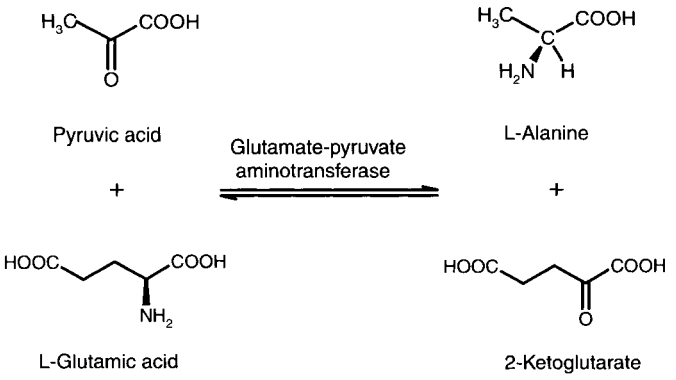

Figure 12.7-3. Transamination using glutamic-pyruvic aminotransferase.

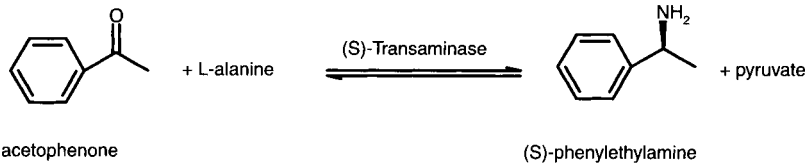

Figure 12.7-4. Enantiomerically pure (*S*)-amines via ω-transaminases.

and L-glutamate over a three-month period with less than 40% loss in activity. Volumetric productivity was 200 $gL^{-1}h^{-1}$ of L-alanine.

12.7.3.2
Synthesis of Enantiomerically Pure Amines

While most methods for the synthesis of enantiomerically pure amines have employed kinetic resolution with the help of lipases or esterases, a method independent of kinetic resolution has been developed using the transamination of ketones catalyzed by ω-transaminases (ω-TA), shown in Fig. 12.7-4 with acetophenone as an example[20, 21].

The ω-transaminases can be employed in two ways to produce both enantiomers in a pure form[18]:

- a racemic mixture can be separated, by kinetic resolution, into the corresponding ketone and the remaining amine enantiomer, which is typically obtained in high enantiomeric excess, the ketone can be recycled as a starting material for the racemic amine;
- the same ω-transaminase can be employed to synthesize the enantiomer of the opposite configuration straight from the ketone.

Both (*R*)- and (*S*)-aminotransferase have been employed at Celgene for the synthesis of enantiomerically pure amines from racemic amines. Degrees of conversion were at or close to 50% for resolutions and enantioselectivities normaly reached > 99% e.e. for the amine product from both resolutions or syntheses from ketones[18, 19]. The donor for resolutions of amine racemates was usually pyruvate whereas either isopropylamine or 2-aminobutane served as donors for reduction of ketones. The amine products ranged from phenylethylamine and tetramines with the amine group at activated benzylic sites or in a cyclic structure, to phenylisoproylamines (amphetamines) or phenoxyisopropylamines where the amine group is hardly or not activated at all. A selection of products synthesized with ω-aminotransferase technology is shown in Fig. 12.7-5. The Celgene process has been scaled up to the 500 kg level[19].

The (*S*)-ω-TA from *Vibrio fluvialis* was found to catalyze the reduction of acetophenone to (S)-α-methylbenzylamine with the concomitant oxidation of L-alanine to pyruvate. The enantiomeric excess was always > 99% e.e. As thermodynamic equilibrium strongly favors the reverse reaction, however, high yields were achieved only when an excess of acetophenone was added and upon removal of pyruvate:

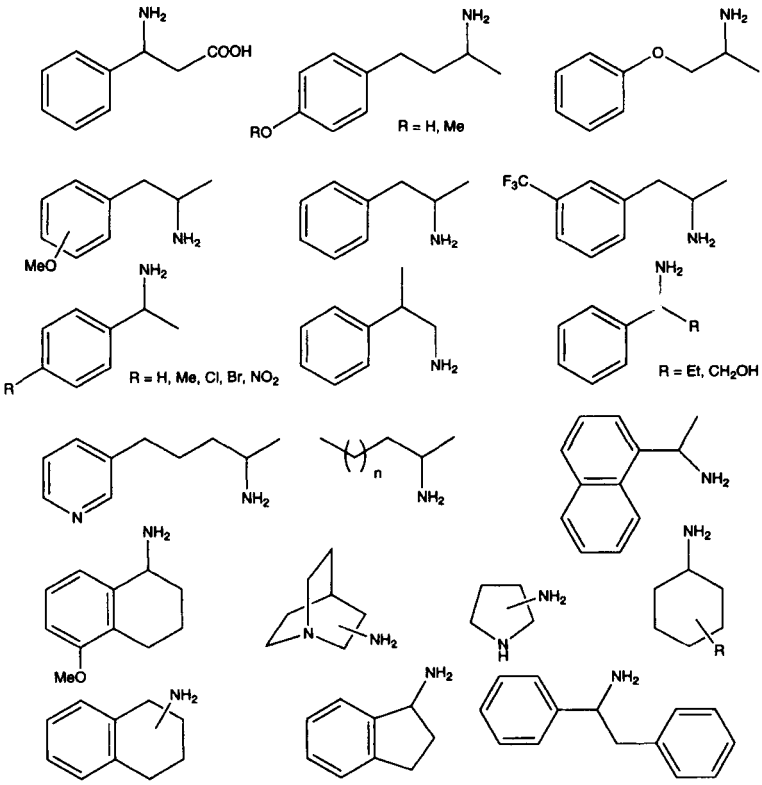

Figure 12.7-5. List of amines produced by ω-aminotransferase technology (both enantiomers produced in each case).

yields of > 90 % were achieved with acetophenone and benzylacetone in the presence of a 10-fold excess of L-alanine if pyruvate was removed by using whole cells. The reaction suffers from strong inhibition by both products, pyruvate and (*S*)-α-methylbenzylamine[20]. Interestingly, the authors found a linear correlation between the reactivities of amino acceptors and the inverse reactivity of amino donors[21].

12.7.3.3
Other Preparative Applications of Aminotransferases

12.7.3.3.1 Preparative Applications: L-Phosphinothricin

L-Phosphinothricin, the active ingredient of the broad-spectrum herbicide Basta (AgrEvo), can be obtained through enzymatic transamination of the corresponding oxoacid, 2-oxo-4-[(hydroxy)(methyl)phosphinoyl]butyric acid, in a coupled system with aspartate aminotransferase (AAT) and 4-aminobutyrate:2-ketoglutarate transaminase (E.C. 2.6.1.19) from *E. coli* (Fig. 12.7-6)[37]. In solutions containing 10 % substrate, 85 % conversion was reached with only < 3 % amino acid by-products. For

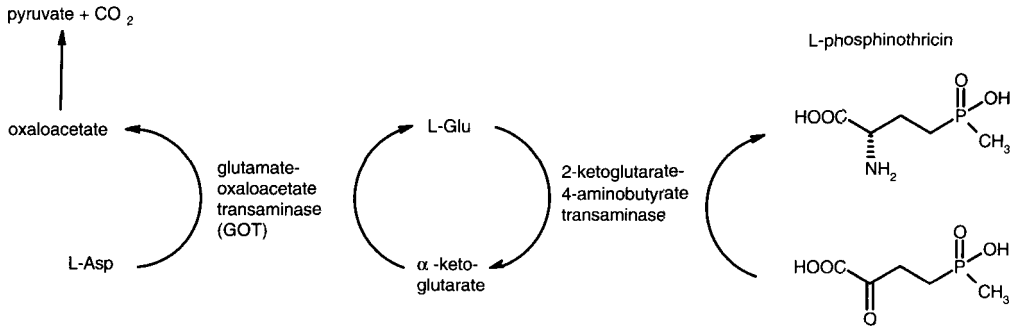

Figure 12.7-6. Coupled process for the herbicide ingredient L-phosphinothricin with transaminases.

this process, a new AAT from *B. stearothermophilus* has been screened and characterized (T_{opt} = 95 °C, pH_{opt} = 8.0) before being cloned and overexpressed in *E. coli*.

12.7.3.3.2 Synthesis of an Omapatrilat Building Block with L-Lysine ε-Aminotransferase

ε-oxo-L-norleucine acetal is a key intermediate for the synthesis of Omapatrilat (BMS-186716), a novel dual-action vasopeptidase inhibitor under development at Bristol-Myers-Squibb (BMS). The BMS researchers developed a novel synthesis of a key building block of Omapatrilat, the bicyclic compound BMS-199541–01, by oxidation of the ε-group of L-lysine in the *N*-protected dipeptide N-Cbz-L-homo-cys-L-lys with a newly found L-lysine ε-aminotransferase[38]. The enzyme was isolated from *Sphingomonas paucimobilis* and was cloned and overexpressed in *E. coli* (2 kUL^{-1}). It is an 81 kDa homodimer with a specific activity towards the product BMS-199541–01 of 1.68 Umg^{-1} of protein; the enzyme requires α-ketoglutarate as a co-substrate which is recycled back into the process after oxidation of the L-glutamate back to α-ketoglutarate by glutamate oxidase (isolated from *Streptomyces noursei*). L-Lysine ε-aminotransferase was found to be one of the most important rate-limiting enzymes in cephalosporin biosynthesis[39].

The process scheme (Fig. 12.7-7) starts from the *N*-protected dipeptide dimer [L-lys-L-homocys]$_2$ disulfide which, after reduction of the S - S bond, is oxidized enzymatically to *N*-Cbz-L-homo-cys-L-lys-ε-aldehyde. Under acidic conditions, the aldehyde group is present as a gem-diol, attacks the α-*N* and closes the ring to the aminol. After nucleophilic attack of the S - H group, the hydroxyl group acts as a leaving group and affords closure of the 1,3-thiazepine ring.

In the biotransformation process to BMS-199541–01, yields of 65–70 mole-% were achieved; without recycling of the L-glutamate resulting from the reduction of α-ketoglutarate yields were substantially lower. L-Lysine ε-aminotransferase also catalyzes the oxidation of *N*-α-protected L-lysines as well as L-lysine peptides such as *N*-protected L-met-L-lys.

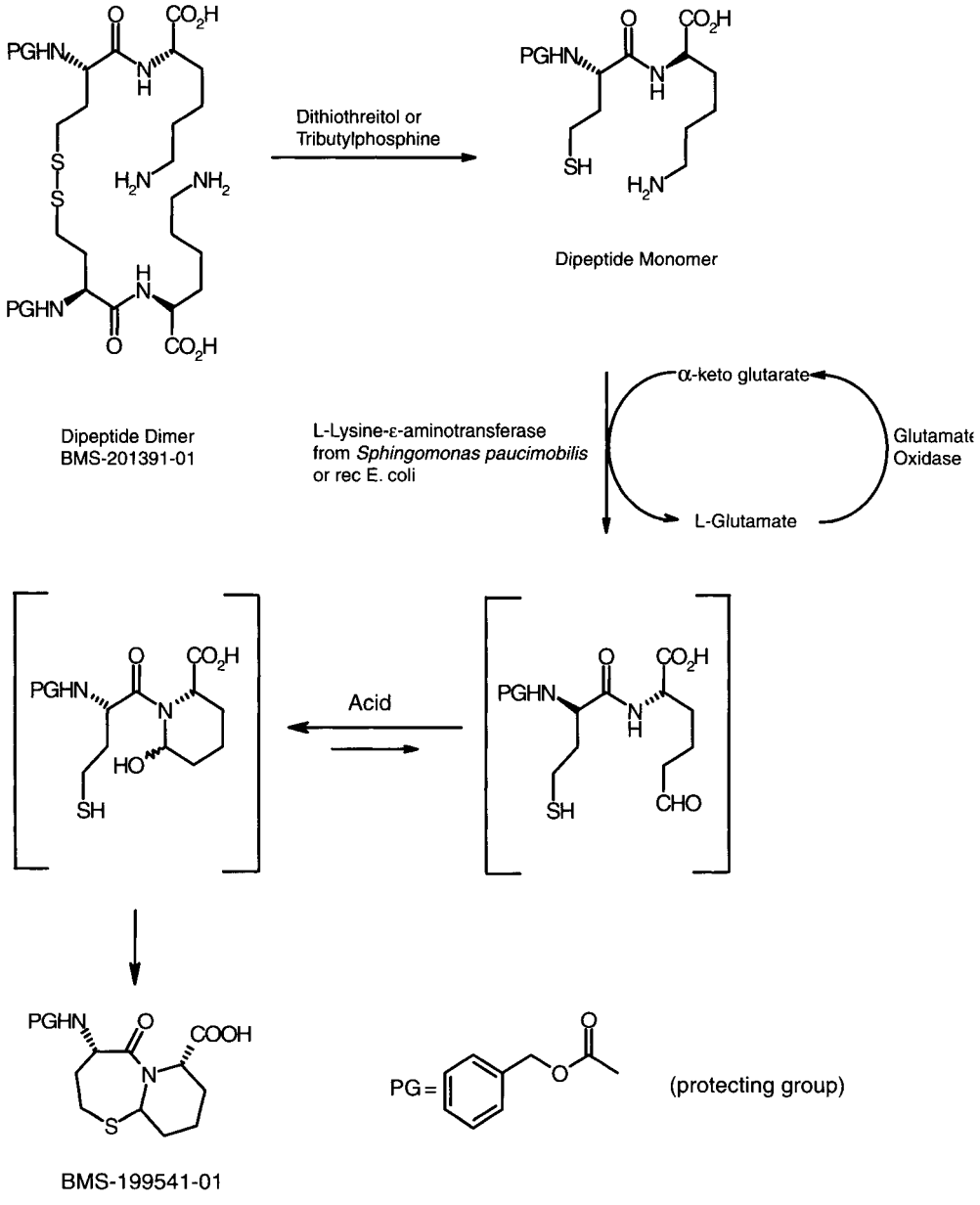

Figure 12.7-7. BMS process to the bicyclic intermediate BMS-199541–01 via L-lysine ε-aminotransferase[35].

12.7.4
Driving the Reaction to Completion

There is one major disadvantage to most of the transamination technology as presented above: because the transamination reaction involves an amino acid reacting with a 2-keto acid to generate products which consist of a 2-keto acid and an amino acid, the equilibrium constant is often close to unity. As a result, the net conversion of substrates to products is thermodynamically limited. The key to the development of an efficient transamination technology lies in overcoming the problem of incomplete conversion of the 2-keto acid precursor to the desired amino acid product.

One approach to this problem is the coupling of the transamination reaction to a second reaction that consumes the keto acid by product in an essentially irreversible step; this drives the transamination reaction to completion. By using an amino-transferase that can utilize aspartic acid efficiently as the amino group donor (instead of glutamic acid), the corresponding 2-keto acid by product is oxaloacetate (rather than 2-ketoglutarate). Oxaloacetate is a β-ketoacid and can be easily decarboxylated to pyruvate. This decarboxylation occurs spontaneously in aqueous solution, catalyzed

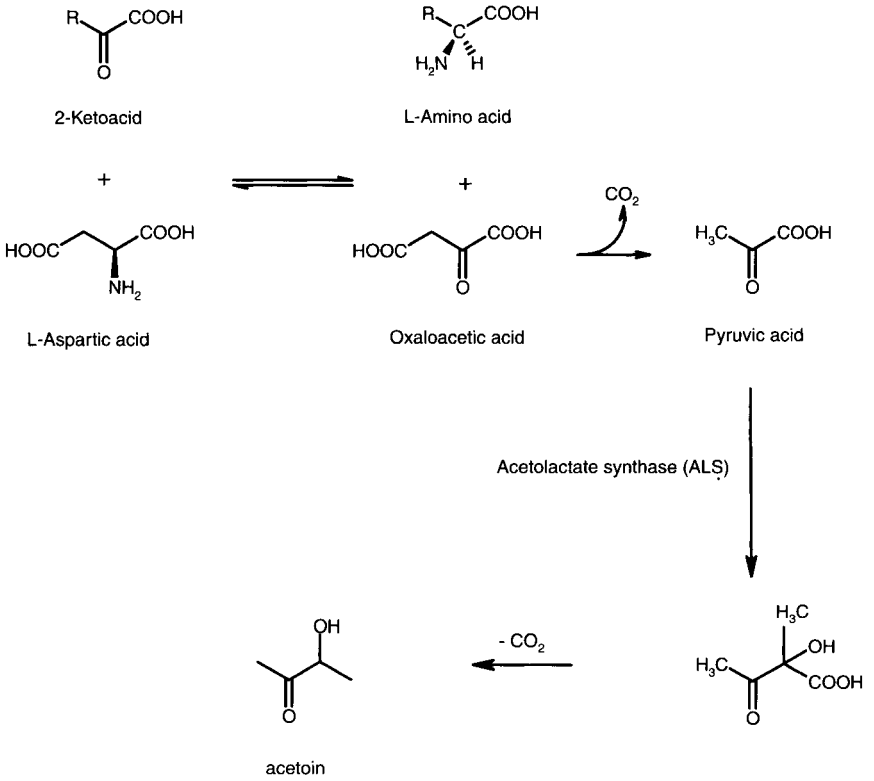

Figure 12.7-8. Driving the transamination reaction to completion.

Table 12.7-1. Time course for the transamination of phenylpyruvate to L-phenylalanine in the presence and absence of oxaloacetate decarboxylase.

Reaction time (min)	Transaminase alone Phenylpyruvate (mM)	Transaminase & Oxaloacetate decarboxylase Phenylpyruvate (mM)
0	200	200
10	184	140
20	166	116
45	124	44
80	116	6

by various metal ions and amines, and can be accelerated chemically, as shown in Fig. 12.7-8, or enzymatically using the enzyme oxaloacetate decarboxylase. The important feature of the process is that the essentially irreversible decarboxylation of oxaloacetate to pyruvate drives the entire process to completion, allowing the transamination of 2-keto acids to amino acids in yields approaching 100% of the theoretical[7, 8, 12, 13]. Importantly, this method of driving the reaction to completion may be used for the production of either D-amino acids or L-amino acids.

The decarboxylation reaction catalyzed by the enzyme oxaloacetate decarboxylase has been examined using enzymes from four different sources: *Pseudomonas putida, Micrococcus luteus,* and two strains of *Azotobacter vinelandii.* The highest rates were obtained with the oxaloacetate decarboxylase isolated from *Pseudomonas,* a Mg^{2+}-requiring enzyme[7, 12].

The effectiveness of decarboxylation in driving the reaction to completion was demonstrated in a coupled enzymatic process by using phenylpyruvate as the starting 2-keto acid. In this experiment, phenylpyruvate sodium salt and L-aspartate were incubated with *E. coli* broad-range transaminase at room temperature and pH 7.5 in both the presence and absence of oxaloacetate decarboxylase from *Ps. putida.* Magnesium ion, which is cofactor for the decarboxylase, was also present in both reaction mixtures at a concentration of 6 mM. The transamination reaction was monitored by following the disappearance of phenylpyruvate. The results are summarized in Table 12.7-1. As demonstrated by the data, when oxaloacetate decarboxylase was included in the mixture the reaction proceeded to completion much more rapidly than in the case when the decarboxylase was omitted[12].

Other methods can also be used for driving the transamination reaction to produce amino acids in high yields. For example, if L-lysine or L-ornithine are used as the donor in the two-enzyme process shown in Fig. 12.7-9, the cyclization of the aldehyde is strongly favored, creating an essentially irreversible reaction that can lead to high yields of a desired amino acid from the corresponding 2-keto-acid[10, 40, 41].

12.7.5
Production of L-Amino Acids Using Immobilized Transaminases

Continuous decarboxylation of oxaloacetate as it is formed is an important part of an efficient, high-yielding transamination process. This decarboxylation occurs readily

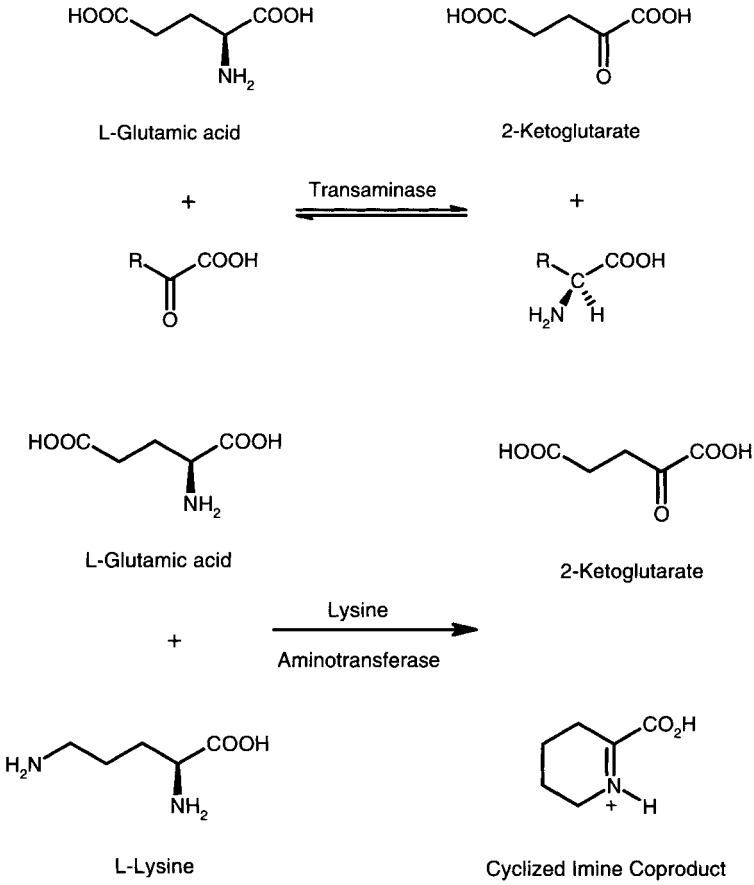

Figure 12.7-9. Coupled reactions using L-lysine for driving the transamination of 2-ketoacids to amino acids.

in aqueous solution, and may be accelerated enzymatically as described above, or chemically using a metal ion such as Mg^{2+} in sufficient concentration. Immobilization of the enzyme allows reuse of the enzyme or continuous production of amino acid in a flow reactor system.

Immobilization of the *E. coli* broad-range transaminase has been accomplished by covalent attachment using glutaraldehyde PVC-silica support matrix that had been activated with a polyamine[13]. In the example described in Table 12.7-1, 4 L of cell lysate containing 61.6 g of enzyme (activity of 5.2 million international units) were clarified by centrifugation at 13 000 *g* for 30 min and recirculated through a pre-activated support matrix for 1.5 h. After washing, 57 g or 93% of the enzyme remained bound to the support. Bound activity was 4.2 million units. The retained activity of the enzyme after immobilization was approximately 89%.

The pH-rate profile for the reaction catalyzed by the *E. coli* broad-range transaminase was determined using the immobilized transaminase with *p*-fluorophe-

Table 12.7-2. Concentrations of reactants for the production of L-*p*-fluorophenylalanine by transamination of *p*-fluorophenylpyruvate.

Reactant	Concentration (mM)
Sodium *p*-fluorophenylpyruvate	100
L-Aspartate	110
Pyridoxal phosphate	0.1
$MgCl_2$	50

nylpyruvate as the keto acid and L-aspartate as the amino group donor. The transamination reaction displayed a fairly broad useful pH range; the immobilized transaminase had a pH optimum of approximately 7.5, but retained activity in the range of pH 6.0–9.5. At pH 5.0 and 10.0, activity fell to less than 20% of that measured at pH 7.

For continuous production of L-*p*-fluorophenylalanine, a typical set of operating conditions is shown in Table 12.7-2. L-Aspartate is used at a 10% molar excess to the starting 2-ketoacid. The cofactor pyridoxal phosphate is added to the reaction mixture to achieve a final concentration of 0.1 mM. The initial pH of the feed solution is 7.2. Mg^{2+} ion was used to accelerate the decarboxylation of oxaloacetate to pyruvate. The reaction was maintained with a temperature range of 37–40 °C. Under these conditions using an immobilized broad-range aminotransferase, the volumetric productivity of the reactor for the production of L-phenylalanine at 85% conversion was 20 $gL^{-1}h^{-1}$.

One of the main advantages of the transamination system is its applicability to a range of other L-amino acids, including non-naturally occurring amino acids. For example, broad-range aminotransferase (encoded by the *aspC* gene) will efficiently transaminate the 2-keto acids corresponding to L-phenylalanine, *p*-fluoro-L-phenylalanine, L-tyrosine, *m*-hydroxy-L-phenylalanine, L-tryptophan, L-methionine, L-homophenylalanine, L-2-aminoadipic acid and a number of others. Using other aminotransferases, the transamination of other 2-ketoacids to the corresponding amino

Table 12.7-3. Amino acids produced by transamination.

Amino Acid	Aminotransferase
L-Phenylalanine	Broad-range, aromatic
L-Tyrosine	Broad-range, aromatic
L-Tryptophan	Broad-range, aromatic
L-*p*-fluorophenylalanine	Broad-range
L-*meta*-tyrosine	Broad-range
L-Homophenylalanine	Broad-range, aromatic
L-2-Aminoadipic acid	Broad-range
L-2-Aminopimelic acid	Broad-range
L-Valine	Branched-chain
L-Leucine	Branched-chain
L-*tert*-leucine	Branched-chain
D-Alanine	D-broad-range
D-Leucine	D-broad-range
D-Tyrosine	D-broad-range
D-Phenylalanine	D-broad-range

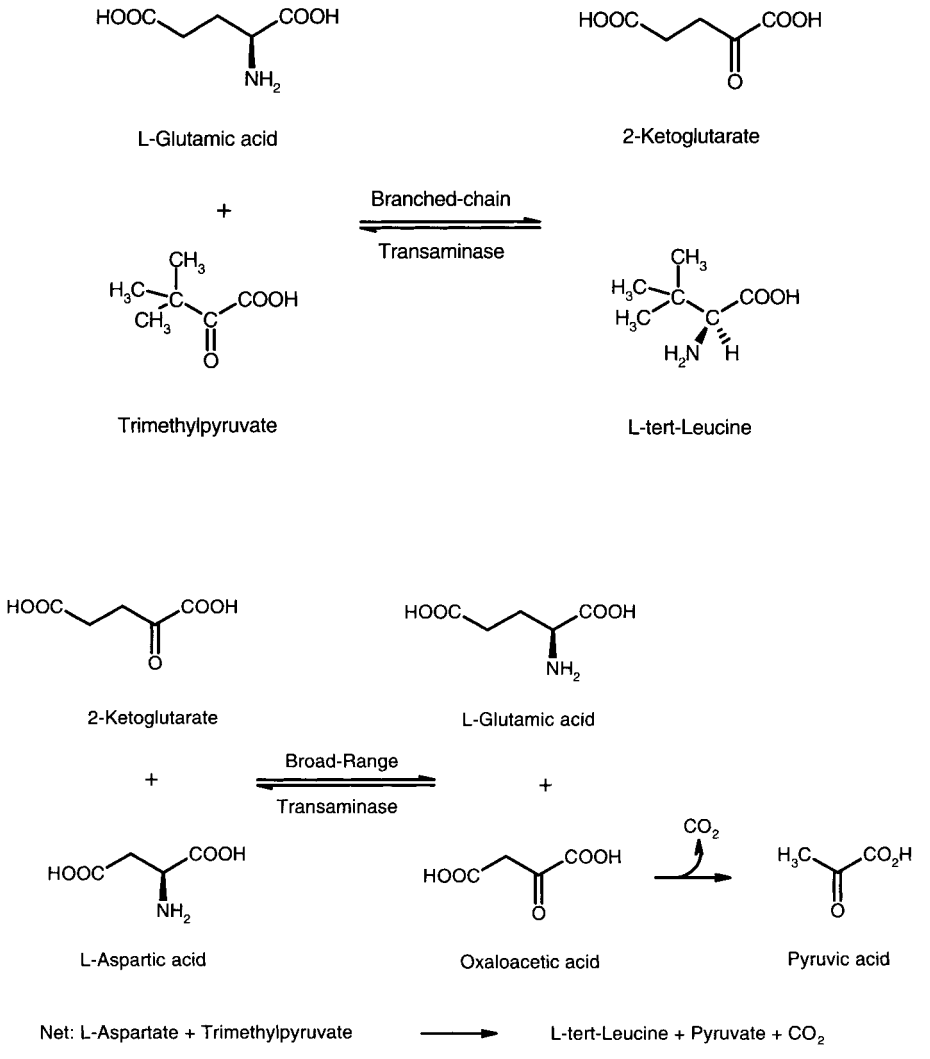

Figure 12.7-10. Coupled aminotransferases for the production of L-*tert*-leucine.

acids can be carried out. A list of amino acids that have been produced by transamination is shown in Table 12.7-3.

The broad-range aminotransferase has low catalytic activity for the group of branched-chain amino acids, including L-leucine, L-isoleucine and L-valine. To enable production of this group of L-amino acids, another transaminase, the so-called branched-chain amino acid transaminase (BCAT), has been used. This enzyme has also been shown to catalyze the transamination of trimethylpyruvate to produce the commercially interesting unnatural amino acid L-*tert*-leucine, although the rate of the reaction is significantly less than that for L-valine. Unlike the broad-range transaminase, the branched-chain aminotransferase is not active with L-

aspartate as the amino donor. L-Glutamate is used for efficient transamination using this enzyme.

To drive this reaction, a coupled transamination reaction was established with both the broad-range and branched-chain aminotransferases acting together as shown in Fig. 12.7-8 for the production of L-*tert*-leucine. In the first reaction, the branched-chain aminotransferase catalyzes the reaction of L-glutamate with trimethylpyruvate to produce L-*tert*-leucine and 2-ketoglutarate. The second reaction catalyzed by broad-range aminotransferase converts L-aspartate and 2-ketoglutarate into oxaloacetate and L-glutamate. The donor L-aspartate is present in stoichiometric amounts relative to 2-ketoisovalerate and is used to continuously recycle the 2-ketoglutarate formed in the first step to L-glutamate as the reaction proceeds. Oxaloacetate is decarboxylated to pyruvate in an essentially irreversible reaction, driving the entire sequence of reactions to completion. The net reaction is the transamination of trimethylpyruvate to L-*tert*-leucine with L-aspartate using 2-ketoglutarate as an intermediary amino transfer agent. This sequence of reactions has also been used to produce L-leucine and L-valine in the laboratory (Fig. 12.7-10).

In laboratory-scale experiments, solutions containing 200–600 mM keto acid were transaminated to the corresponding branched-chain L-amino acid, with a concentration of L-glutamate between 50 mM and 100 mM and a 1.1 molar excess of L-aspartate. Yields obtained for the branched-chain amino acids have typically been in the range of 80–90% based on starting with a 2-keto acid[10].

Another example of a coupled enzyme reaction demonstrates the versatility of the transaminase system in biocatalysis. Using a racemic D,L-amino acid mixture as the starting material, the enzyme D-amino acid oxidase from *Trigonopsis variabilis* will convert the D-amino acid in the mixture selectively into the corresponding 2-keto acid. The L-amino acid of the D,L- pair is neither a substrate nor an inhibitor of D-amino acid oxidase. If a transaminase is present in the same reaction mixture, the 2-keto acid can be transaminated in the presence of L-aspartate to the corresponding L-amino acid. The entire reaction can be driven to completion as described previously by decarboxylation of the oxaloacetate. Thus, in a single pot, racemic D,L-amino acids can be convened directly into optically active L-amino acids (Fig. 12.7-11).

12.7.6
D-Amino Acid Transferases

The aminotransferase reaction can be utilized for the synthesis of D-amino acids as well as the better-known route to L-amino acids (Fig. 12.7-11). Regarding sequence similarity, D-aminotransferases form a distinct subgroup among the transferases, however, it has been found, with the help of crystal structures[42–43] that some striking similarities exist between L-amino acid aminotransferases with respect to active site structure and to branched-chain aminotransferase (BCAT) with respect to sequence. D-Aminotransferases utilize the same PLP chemistry as L-aminotransferases to effect transamination[42]. Mutagenesis of a distant interdomain loop of D-aminotransferase to produce enhanced conformational flexibility (pro119-arg120-

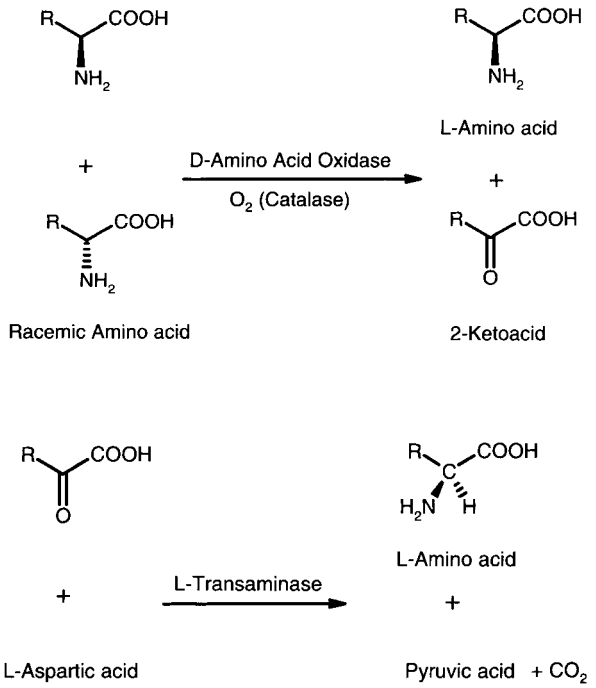

Figure 12.7-11. Conversion of racemic amino acids into L-amino acids with D-amino acid oxidase and an L-aminotransferase.

pro121 to gly-gly-gly) resulted in higher catalytic constants towards most D-amino acid substrates[44].

D-Amino acids can also be produced directly by transamination using a D-aminotransferase. Since these enzymes require a D-amino acid donor, we developed a coupled enzymatic reaction with aspartate racemase to generate D-aspartic acid *in situ* from inexpensive L-aspartic acid. The reaction scheme is shown in Fig. 12.7-12. Aspartate racemase, cloned from *Streptococcus thermophilus* and expressed in *E. coli*, is used in conjunction with a D-aminotransferase to produce D-amino acids from corresponding 2-ketoacids in a reaction that is analogous to that for the production of L-amino acids. Oxaloacetate, produced from D-aspartate during the transamination, is decarboxylated to pyruvate, driving the reaction to completion as with the L-transamination. Significant amounts of D-alanine are produced using the D-aminotransferase cloned from *Pseudomonas sphaericus* ATCC 10 208 as it has activity toward pyruvate. Directed evolution efforts are in progress to develop an enzyme having reduced D-alanine production, resulting in a cleaner product mixture. For the synthesis of D-glutamate, a two-enzyme system consisting of of glutamate racemase and D-aminotransferase has been found in *B. sphaericus*[45].

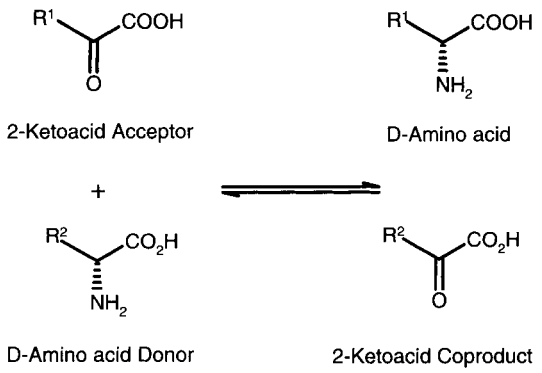

Figure 12.7-12. Production of D-amino acids by transamination.

12.7.7
Synthesis of Labeled Amino Acids

Isotopically labelled amino acids are particularly amenable to production by transamination. Because the reaction catalyzed by aminotransferases transfers a specific amino group from the donor, amino acids highly enriched in isotopes such as ^{15}N can be produced. For example, ^{15}N L-tyrosine has been produced in greater than 90% yield from ^{15}N L-aspartate and *p*-hydroxyphenylpyruvate using the broad-range aminotransferase from *E. coli*. The reaction is shown schematically in Fig. 12.7-13. Analysis of ^{15}N-isotope incorporation was carried out by mass spectrometry by Cambridge Isotope Laboratories. The samples showed incorporation of 98.4% ^{15}N, which was almost identical to the isotopic purity of the starting L-aspartic acid. Only the L-isomer of tyrosine was detectable by chiral HPLC. This result established the feasibility of the production of ^{15}N amino acids by transamination by meeting three important criteria for success:

- there was no detectable loss of isotopic purity in the transfer of the amino group from ^{15}N aspartic acid to the 2-ketoacid,

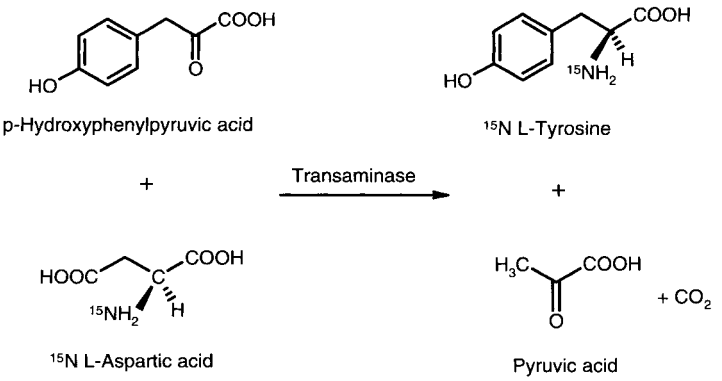

Figure 12.7-13. Production of ^{15}N-labeled amino acids by transamination.

- the stereochemical fidelity of the transamination reaction was perfect within detection limits, and
- The yield of conversion of the ^{15}N aspartic acid (the most costly starting material in this reaction) was high (> 90%).

12.7.8 Availability of Enzyme

In order to facilitate the production of adequate amounts of transaminase at low cost, the genes encoding aminotransferases have been cloned and overexpressed in *E. coli*. Two examples are the *aspC* and *ilvE* genes from *E. coli*. The expression of these genes has been described previously, with the levels of aminotransferase enzyme reaching approximately 30–40% of the total cell protein [12]. More recently, the genes encoding other aminotransferase genes have been cloned [46–50], leading to the availability of a broader group of aminotransferase enzymes for evaluation. Given the high reaction rates observed and the potential for wide applicability for the production of amino acids, both D and L, natural or unnatural, transamination reactions should prove to be useful method for the chemist.

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13
Formation and Cleavage of P–O Bonds

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13.1
Introduction

The use of isolated enzymes to form or cleave P–O bonds is an important application of biocatalysts. Restriction endonucleases, (deoxy)ribonucleases, DNA/RNA-ligases, DNA-RNA-polymerases, reverse transcriptases etc. are central to modern molecular biology[1]. Enzyme catalyzed phosphoryl transfer reactions have also found important applications in synthetic organic chemistry. In particular, the development of convenient cofactor regeneration systems has made possible the practical scale synthesis of carbohydrates, nucleoside phosphates, nucleoside phosphate sugars and other natural products and their analogs. This chapter gives an overview of this field of research.

Hundreds of potentially useful enzymes are available in nature. It is often worthwhile to survey enzymes for applicability in the synthesis of a specific compound, but how to find the best enzyme? Enzymes have been reviewed and classified by many schemes[2–4]. Enzymes involved in reactions at phosphoryl groups are, unfortunately for the synthetic chemist, spread almost over all classes. Without a good knowledge of enzymology, it is not easy to find the enzyme classes of interest for a particular transformation. This review links the compound classes and enzyme classification systems in Section 13.1.1 to help overcome this barrier.

Most synthetically useful phosphorylating enzymes require nucleoside triphosphates as cofactors. The central importance of cofactor regeneration, and the most used regeneration methods for these cofactors, are discussed in Section 13.2.1. The end of Chapter 13 includes tabular surveys of the most important applications, classified in compound or structural classes (see Sections 13.2.2 and 13.3.3), to facilitate the search for relevant enzymes and procedures.

13.1.1
Enzymes Forming or Cleaving Phosphorous-Oxygen Bonds

Phosphoesters are ubiquitous in biochemistry and serve several functions [5]. Genetic information is stored in DNA and RNA. In cellular control mechanisms, phosphorylation of proteins is an important mechanism for regulating protein activities [6]. Phosphorylation can activate metabolites or change solubility properties. Enzyme-catalyzed formation and cleavage of P–O bonds are central to the cellular energy balance [7]. Biosynthesis depends heavily on phosphorylated intermediates.

A useful classification for enzymes involved in phosphoryl transfers was introduced by Knowles [8] (see Fig. 13-1). This classification, based on enzyme functions and mechanisms, differentiates primarily between two groups of enzymes. The first group contains only enzymes that accept phosphoric monoesters as substrates (type A and B). The second group includes all enzymes catalyzing reactions at phosphoryl groups of phosphodiesters (type C–E). Table 13-1a and 13-1b link Knowles' classification and the enzyme classification recommended by the International Union of Biochemistry (IUB; compare Chapter 1) [2]. The IUB classes give a direct access to the specific enzymes in reference works and to the CA registry numbers necessary for an efficient literature search [2, 4].

Tables 13-1a and 13-1b list only the most important categories of enzyme classes (E.C.'s). Some enzymes that are involved in reactions at phosphorus are hidden in other classes. For example glyceraldehyde-3-phosphate dehydrogenase, which catalyses the oxidative phosphorylation of glyceraldehyde-3-phosphate to 1,3-diphosphoglycerate, is classified under E.C. 1.2.1.12 and 1.2.1.13. Neither the name of the enzyme nor its IUB-classification, gives information about the phosphorylating step. Identifying enzymes potentially useful in synthesis that have been ambiguously classified is difficult for those outside of biochemistry because no complete reference is available connecting enzymatic activity with synthetic applicability.

A second important point is that many enzyme catalyzed reactions are reversible. Some hydrolytic enzymes can be used in enzyme catalyzed phosphorylation reac-

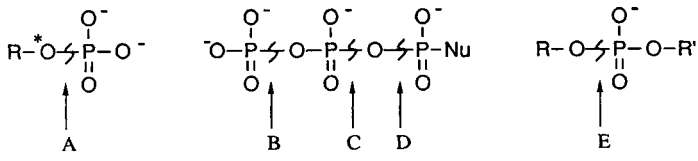


Figure 13-1. Classes of enzymes involved in reaction at phosphorus. A and B represent enzyme types that handle phosphoric monoesters and related compounds (*O may be an oxygen of a hydroxyl, carboxyl, or phosphoryl group, or the nitrogen of a guanidine group. For simplicity, displacements at the γ-phosphoryl groups of nucleosides triphosphates were classified with these reaction). C, D and E represent the enzymes that catalyze transformations of phosphoric diesters (displacements at α or β phosphorous groups of nucleoside triphosphates and transfer of pyrophosphates were classified with the reactions of phosphoric diesters).

Table 13-1a. Enzymes accepting phosphoric monoesters as substrates.

Enzyme type[a]	Functional class[b]	Function[c]	IUB classes with titles, containing such types of enzymes[d]
A	Phosphomutases	Phosphoryl group transfer, for which the acceptor is another functional group on the donor molecule.	2.7.5. Phosphomutases 5.4.2. Intramolecular phosphotransferases
A	Phosphorylases	Formation of a P–O bond under phosphorolytic cleavage of a C-Heteroatom bond.	2.4.1. Hexosyltransferases 2.4.2. Pentosyltransferases
A	Nucleotidases	Phosphoryltransfer from a nucleotide to water as an acceptor molecule. (Nucleotides are cleaved hydrolytically).	3.1.3. Phosphoric ester hydrolases (3.1.4. Phosphoric diester hydrolases)
A/B	Phosphatases	Phosphoryl group transfer from a phosphoric monoester to water as an acceptor molecule. (Phosphoric monoesters are cleaved hydrolytically).	3.1.3. Phosphoric ester hydrolases 3.6.1. Hydrolases acting on acid anhydrides in phosphorous-containing anhydrides
A/B	Phosphokinases and Phosphotransferases	Phosphoryl group transfer: Nucleoside triphosphate is the donor and some other molecules than H_2O are the acceptors. Compounds different than nucleoside triphosphates are the donor and some other molecules than H_2O are the acceptors.	2.7.1. Phosphotransferases with an alcohol group as acceptor 2.7.2. Phosphotransferases with a carboxyl group as acceptor 2.7.4. Phosphotransferases with a phosphate group as acceptor
B	ATPases	Phosphatases which are responsible for the coupling of ATP cleavage to other metabolic processes.	3.6.1.3 ATPases

a See figure 13-1; **b** functional classes bases on ref.[8]; **c** see ref.[8] and [4]; **d** see ref.[2].

tions. Alkaline phosphatase (E.C. 3.1.3.1), for example, was used in enzyme-catalyzed phosphorylation of glycerol with inorganic phosphate[9]. In some cases enzymes may catalyze unexpected reactions with unnatural substrates: aminoacyl tRNA synthetases (ARS) were used to synthesize p^1,p^4-di(adenosine 5'-)tetraphosphate (Ap_4A; **1**), a natural inhibitor of human platelet aggregation[10] (Fig. 13-2). Here, in the first step an amino acid (AA) reacts reversibly with ATP and ARS and forms an aminoacyl-AMP-ARS complex and PP_i; the back reaction of this intermediate with ATP leads to the desired product Ap_4A[11–13].

Table 13-1b. Enzymes accepting phosphoric diesters as substrates.

Enzyme type[a]	Functional class[b]	Function[c]	IUB classes with titles, containing such types of enzymes[d]
C	Pyrophosphokinases	Pyrophosphate group transfer from ATP to an acceptor molecule other than water.	2.7.6. Diphosphotransferases
D	Nucleotidyl transferases	Transfer of nucleotidyl moieties	2.7.7. Nucleotidyltransferases
D	Nucleotidyl cyclases	Nucleoside triphosphate cyclisation under formation of pyrophosphate	4.6.1. Phosphorous oxygen lyases
E	Triphosphohydrolases	Triphosphate transfer from a nucleoside triphosphate to water as an acceptor molecule.	3.1.5. Triphosphoric monoester hydrolases
E	Polynucleotide synthetases	Responsible for the linkage of two poly- or oligonucleotide moieties to form polynucleotide chains	6.5.1. Ligases forming phosphoric ester bonds
E	Phospholipases	Hydrolytic cleavage of phosphoglycerides (essentially phospholipase C and D)	3.1.4. Phosphoric diester hydrolases
E	Nucleases	Phosphonucleotide transfer from a polynucleotide to water as an acceptor molecule. (Polynucleotides are cleaved hydrolytically).	3.1.4. Phosphoric diester hydrolases 3.1. Endo- and exonucleases
E	Phosphodiesterases	Phosphomonoester transfer from a phosphodiester other than polynucleotide to water as an acceptor molecule. (Phosphodiesters are cleaved hydrolytically).	2.7.8. Transferases for other substituted phosphate groups 3.1.4. Phosphoric diester hydrolases

a See figure 13-1; **b** functional classes bases on ref.[8]; **c** see ref.[8] and [4]; **d** see ref.[2].

One of the most important criteria in the evaluation of a new process is the availability of an enzyme[14] (see Chapter 20: Tabular Survey of Commercially Available Enzymes). If the enzymes are not commercially available, their isolation and purification can be expensive and time consuming (see Chapter 2: Production and Isolation of Enzymes). The importance of the product to be synthesized may sometimes justify the additional effort.

Mechanistic aspects of P–O bond formations and cleavages have been reviewed[15] and are outside the scope of this work. The use of enzymes catalyzing the formation

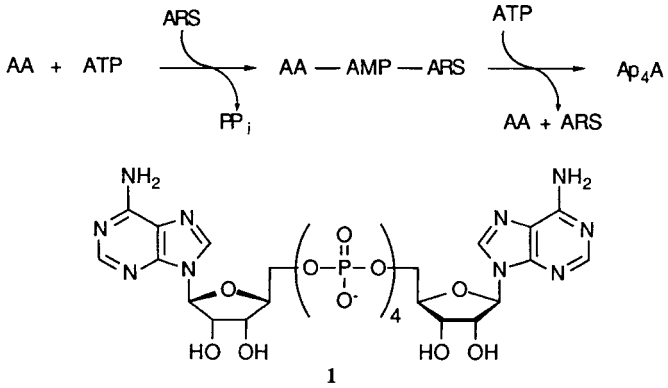


Figure 13-2. Enzymatic synthesis of p^1,p^4-di(adenosine 5'-)tetraphosphate (Ap_4A; **1**) with aminoacyl tRNA synthetases (ARS). AA can be leucine, for example, and ARS leucyl t-RNA synthetase[11].

of P–N bonds – for example, phosphorylations of amino acids (E.C. 2.7.3) – are discussed only briefly. Enzymes dealing with the formation of aminoacyl tRNA (E.C. 6.1.1), acyl-CoA derivatives (E.C. 6.2.1) or peptides (E.C. 6.3.2) are also not covered, even if cleavages of nucleoside phosphates are involved.

13.1.2
Biological Phosphorylating Agents

To compare the ability of different compounds to transfer a phosphoryl group, phosphorylation of water was chosen as a standard reaction[17]. The free energy of hydrolysis of a phosphorus compound ($\Delta G^{o\prime}_{hydr}$) is called its phosphorylating potential. Table 13-2 summarizes the phosphorylating potentials of the most important biological compounds (Fig. 13-3) having phosphoryl donor abilities.

By far the most important strong biological phosphorylating agent is adenosine 5'-triphosphate (ATP, **8**). ATP is ubiquitous and plays a central role as cofactor in anabolic and catabolic processes. Moreover, many enzymes involved in the formation of P–O bonds are ATP dependent. The biologically active form of ATP is, in most cases, the magnesium salt $MgATP^{2-}$[22]. Other nucleoside triphosphates have similar phosphorylating potentials but they are rarely used as phosphoryl group donors[23, 24]; usually GTP, CTP and UTP act as nucleoside or nucleoside phosphate donors (see Section 13.2.2.2).

Creatine- and arginine phosphate (**7** and **9**) play important roles in the storage of phosphorylating potential in vertebrates and invertebrates, respectively[25, 26]. In living cells, these *N*-phosphoguanidine derivatives are formed by phosphoryl group transfer from ATP, and in the reverse reaction ADP is the only acceptor for **7** and **9**.

1,3-Diphosphoglycerate (**5**) and phosphoenolpyruvate (**2**) are important phosphorylating agents of ADP in the glycolytic pathway. Polyphosphate[27], phosphoramidate[28] and pyrophosphate[29] are involved in the biochemical phosphorylation

Table 13-2. Free energies of hydrolysis of some important biological phosphorus compounds[16, 17].

Compound ($R\text{-}OPO_3^{2-}$)	pH	[kcal/mol]	[kJ/mol]	
Phosphoenolpyruvate (**2**)	7.0	12.8	53.5	strong phosphorylating agents
Methoxycarbonyl phosphate[b] (**3**)	7.0	12.4	51.8	
Carbamyl phosphate (**4**)	9.5	12.3	51.4	
1,3-Diphosphoglycerate (**5**)	6.9	11.8	49.3	
Acetyl phosphate (**6**)	7.0	10.3	43.1	
Phosphocreatine (**7**)	7.0	10.3	43.1	
ATP (**8**) (→ ADP + Pi)[c]	7.4	7.3–9.6	30.5–40.1	
ATP (**8**) (→ AMP + PPi)	7.0	7.7	32.2	
Arginine phosphate (**9**)	8.0	7.7	32.2	
Pyrophosphate[c] (PP_i)	7.0	4.5–8.0	18.8–33.4	weak phosphorylating agents
Glucose 1-phosphate (**10**)	7.0	5.0	20.9	
Glucose 6-phosphate (**11**)	7.0	3.3	13.8	
Glycerol-1-phosphate (**12**)	8.5	2.2	9.2	

[a] The standard free energies are bases on a standard state of 1 M total stoichiometric concentration of reactants and products, except hydrogen ion, and on an activity of pure water of 1.0; [b] see ref.[18]; [c] Hydrolysis of ATP and PP_i depend strongly on the concentration of Mg^{2+} in solution and on pH[19–21].

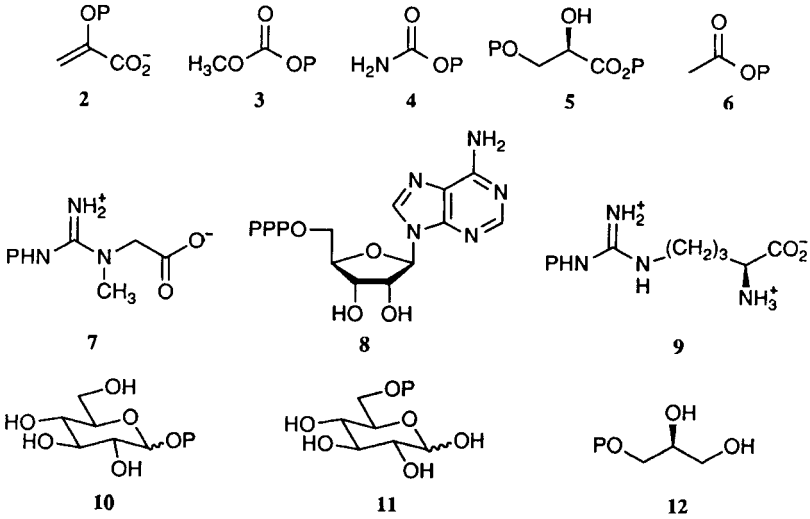


Figure 13-3. Structures of the most important biological phosphorylating agents. P = phosphate.

of D-glucose, hexoses and L-serine respectively in some organisms. Carbamylphosphate (**4**) and acetylphosphate (**6**) have high phosphorylating potentials (see Table 13-2), but nature uses them mainly as donors of carbamyl[30] or acetyl groups[31]. Only in a few cases do they act as phosphoryl donors[30, 32].

Phosphorylations with low-potential phosphorylating agents are thermodynamically not favorable. In biological systems, these processes are made possible by

coupling them to a thermodynamically more favorable process. Examples of weak phosphorylating agents are sugar phosphates such as glucose- and ribose phosphates, which can transfer their phosphate group to other sugars[32] or to nucleosides like riboflavin[33]. Phosphate sugars are formed when polysaccharides are cleaved with a phosphorylase and inorganic phosphate[34].

13.2 Phosphorylation

Chemical phosphorylations usually involve many protection and deprotection steps. Enzymatic phosphorylations can make synthesis more efficient by eliminating many of these steps. In addition, enzyme-catalyzed introduction of phosphoryl groups can be diastereo-[35] or enantiospecific[36, 37].

One of the major challenges in enzyme-catalyzed phosphorylation reactions is, as mentioned above, the choice of the most convenient enzyme. The other major difficulty is the availability of the coenzymes. Cofactors act as biological phosphoryl donors and in enzyme-catalyzed synthesis, they have to be added in stoichiometric amounts or coupled to an efficient regeneration system.

13.2.1 Regeneration of Nucleoside Triphosphates

In enzyme-catalyzed synthesis, adenosine 5'-triphosphate (**8**) is the cofactor most often used as phosphoryl group donor. Other nucleoside phosphates, UTP, or CTP are used principally as donors of a nucleoside phosphate moiety to form activated intermediates in biological pathways (see Section 13.2.2.2). For example: UTP precedes the activated from a glucose, UDP-glucose, in the Leloir synthesis of polysaccharides, CTP precedes CDP-choline in the synthesis of phospholipids and CMP-NeuAc in the formation of glycosides of sialic acids (see Chapter 11.3). The costs for a mole CTP, GTP or UTP vary from $ 32000 to 90000 (as research biochemicals)[38]. The high price of these cofactors precludes their large-scale use in stoichiometric quantities and makes cofactor regeneration necessary. Even with ATP, one of the least expensive cofactors used in organic synthesis[39, 38] and available through mole scale synthesis from RNA[40] regeneration remains of central importance. The use of a cofactor regeneration system not only eliminates the need for stoichiometric quantities of cofactor but it can also favorably influence the position of the reaction equilibrium and prevent the accumulation of cofactor by-products that may inhibit the forward process. Product isolation is simplified as well.

A nucleoside phosphate regeneration system must meet several specifications to be practical. To be economical, a regeneration method must be capable of recycling the cofactor 10^2–10^6 times[39]. All materials should be readily available, inexpensive, easily handled, stable under reaction conditions and compatible with the rest of the reaction system. The transfer of phosphate should be thermodynamically and

kinetically favorable and it should be regioselective in forming a high-energy P – O bond.

13.2.1.1
Regeneration of ATP from ADP and AMP

At the scale required for synthesis of fine chemicals, the major problems of ATP regeneration have been solved[39, 41, 42]. Three strategies have been applied: chemical synthesis; biological methods including whole cells, organelles, and fermentation processes; and cell-free enzymatic catalysis. Chemical methods often lack the necessary specificity and are not compatible with biochemical transformations. Biological and enzymatic systems provide the most efficient ATP regenerating systems[39]. The use of cell-free enzymes requires a greater initial effort or expense than do the biological methods, but are more specific than biological systems and often generate fewer by-products (see ref.[39] and references cited therein).

a) From ADP. Several procedures for the large-scale regeneration of ATP from ADP using isolated enzymes as catalysts are known[43, 39]. These methods have in common the characteristic that phosphoryl groups are transferred from a high-energy phosphoryl donor to ADP (compare Section 13.1.2). The advantages and disadvantages of these methods are summarized in Table 13-3.

In practice, for most synthetic applications, either acetyl phosphate/acetate kinase or phosphoenolpyruvate/pyruvate kinase are used to regenerate ATP. Because of the ease of preparing AcP, AcP/AcK is the most economical method for large-scale work. Its application is, however, limited to fast phosphorylation reactions where the hydrolysis of AcP is not important. The PEP/pyruvate kinase system is used in instances where the requirement for a strong, stable phosphorylating reagent outweighs the relative inconvenience of preparation of PEP.

Phosphoenolpyruvate/pyruvate kinase. Phosphoenolpyruvate (PEP; **2**)/pyruvate kinase (PK; E.C. 2.7.1.40) is the most efficient system for the regeneration of ATP from ADP. The phosphorylating agent PEP can be prepared in a mole scale[47]. Starting from crude pyruvic acid, the crystalline monopotassium salt PEP-K^+ is synthesized in a three-step procedure. For transformations on a scale <1 mol, PEP can be prepared from commercially available 3-phosphoglyceric acid in an enzyme-catalyzed reaction[64]. This method is more expensive than the chemical preparation, but is more convenient because it requires less time and produces less organic waste (see Section 13.2.1.2; Fig. 13-5).

Pyruvate kinases are commercially available from multiple sources[38]. The enzyme generally used in ATP regeneration – from rabbit muscle – has high specific activity (~500 U per mg of protein), is inexpensive ($ 2–4/1000 U), and is stable when immobilized[65–67]. This regeneration system can be used in membrane enclosed enzyme catalysis (MEEC technique)[68, 69] as well.

The stability of PEP in solution and its strength as a phosphoryl donor (Table 13-2) make PEP particularly convenient for use in slow and thermodynamically un-

Table 13-3. Properties of ATP-regeneration systems.

		PEP/PK	AcP/AcK	MCP/AcK	CP/CK	CrP/CrK	P_n/P_nK	P_i/glycolysis
	costs [$/1000 U], (source)[38]	2.5 (rabbit muscle)	378 (*B. stearothermophilus*)	378 (*B. stearothermophilus*)	10 (*Streptococcus faecalis*)	2.6 (rabbit muscle)	isolated from *E. coli*[44]	66 (from diff. sources)[a]
	product inhibition/ K_i [mM][b]	pyruvate 10, C	acetate 400, NC	HCO_3^- 500, NC	c	creatine[25] 6–40[d], NC	–	–
Enzyme properties	spec. activity [U/mg protein][38]	300–500	400–1200	≥ 140[b]	400–900	150–250	150–250[44]	8[h]
	stability	++++[e, 47]	++++[e, 48]	+++[f, b]	+[30]	+++[f, 49]	++++[e, 44]	+[a, f]
	K_m (ADP)[f] [mM]	0.1[b]	0.4[50]	–	0.05[c]	0.05[25]	0.17[44]	–
	K_m (P-donor) [mM]	0.07[b]	0.4[b]	1.6[b]	0.1[c]	5[f, b]	0.003[44]	–
	ease of preparation/ availability	+[47]	+++[5]	++[b]	+++[52]	+[53]	+++++[54]	+++++[38]
P-donors properties	ΔG (P-transfer)[g] [kcal; kJ/mol]	– 5.5; – 23.0	– 3.0; – 12.6	– 5.1; – 21.3	– 5.0; – 20.9	– 3.0; – 12.6	≤ – 0.7; – 2.9[i]	– 16; – 67.5[k]
	half life for hydrolysis (25 °C, pH 7)[b]	~ 10^3	21	0.3	2.2	~ 10^{21}	–	–

a calculated from 1000 U of each enzyme; see ref.[45] and [38]; **b** see ref.[18] C = competitive, NC = non-competitive; **c** carbamate kinase kinetics is complex, inhibition plays an important role[46]; **d** K_i depends strongly on the anions present in solution[25]; **e** immobilized enzyme(s); **f** value(s) for the free enzyme(s); **g** calculation based on the values from Table 13-2 (ΔG(P-transfer) = ΔG^0_{hydr} (P-donor) – ΔG^0_{hydr} (ATP); ΔG^0_{hydr} (ATP) = – 7.3 kcal/mol; 30.5 kJ/mol); **h** calculation based on the sum of all enzymes present; **i** based on Table 13-2, values for PP_i; **k** the driving force of this process is the transformation of glucose to 2 equivalents of lactate (ΔG = – 197 kJ/mol)[22]; **l** calculated from data in ref.[55].

favorable phosphorylation reactions. It is also the method of choice for the regeneration of ATP at low concentrations of ADP, since the Michaelis constant for PK is smaller (K_m(MgADP) = 0.1 mM)[18] than for acetate kinase (K_m(MgADP) = 0.4 mM)[50].

The PEP/PK regeneration method has two minor disadvantages. First, the synthesis of PEP[64, 47] requires more effort and expense than does the synthesis of AcP[57]. Second, pyruvate is a strong inhibitor of PK (see Table 13-3). The reactions are therefore carried out in dilute solutions to keep the pyruvate concentration low, and pyruvate is either removed from the reaction solution or PEP is used at high concentrations to minimize the effects of inhibition.

Acetyl phosphate/acetyl kinase. Acetyl phosphate (AcP; **6**)/acetyl kinase (AcK; EC 2.7.2.1) is the most widely used large scale ATP-regeneration system. AcP is modestly stable in aqueous solutions and is a phosphoryl donor of intermediate strength (Table 13-2 and 13-3). Diammonium acetyl phosphate can be prepared from ketene and anhydrous phosphoric acid[56] or, more easily, from acetic anhydride and anhydrous H_3PO_4[57]. However, the use of the diammonium salt in ATP regeneration has three disadvantages. First, NH_4^+ reacts with acetyl phosphate in solution. Second, it forms an insoluble precipitate with Mg^{2+} under reaction conditions. Third, its preparation involves several steps that require careful experimental control and that are difficult to carry out at large scale. Preparation of aqueous solutions of acetyl phosphate as its sodium[51] or potassium salt[18] circumvents these drawbacks.

Two types of commercially available[38] acetate kinases, from *Escherichia coli*[58] and from *Bacillus stearothermophilus*[48], have been used in ATP regeneration. The latter kinase is more expensive but it is preferred for synthetic use because it is thermostable and it is stable to autoxidation[59]. Both enzymes have acceptable specific activities (150–300 and 400–1200 U respectively per mg protein) and can be stabilized by immobilization[48, 58, 60–62]. Acetate is a weak inhibitor of AcK, but product inhibition is not a serious problem (see Table 13-3) unless reaction solutions have acetate concentrations greater than 1 M[43]. The relative instability of AcP in solution compared with PEP is the major disadvantage of the AcP/AcK system. The contribution of the enzymes to the total cost of the process in generally low when they are recycled, making the slightly higher cost of AcK compared with PK a minor disadvantage[39]. Polymer bound ATP was regenerated in a membrane reactor with AcP/AK[63].

Methoxycarbonyl phosphate/acetate kinase. Methoxycarbonyl phosphate (MCP; **3**) was designed to replace AcP as phosphoryl donor[18]. It is comparable to PEP in its high phosphorylating strength (see Table 13-2), but resembles acetyl phosphate in its ease of synthesis. Aqueous solutions of MCP are prepared from aqueous phosphate and methyl chloroformate and used in ATP regeneration without purification. The reaction product after phosphoryl transfer is methyl carbonate, which hydrolyses rapidly to form CO_2 and MeOH. Product isolation is simple and bicarbonate inhibition can be avoided by purging the reaction mixture.

MCP is accepted as an unnatural substrate by AcK (E.C. 2.7.2.1) and CK (E.C. 2.7.2.2) but not by PK[18]. The principal disadvantage of methoxycarbonyl phosphate as phosphorylating agent in ATP regeneration is MCP's rapid spontaneous decomposition. The half-life of MCP in aqueous solution is only 0.3 h (25 °C, pH 7)[18]. Because of this short half-life, MCP is only used in a few cases where high phosphorylating potentials are required to push the phosphorylation reaction to the product side (see Table 13-4, entry 20).

Others. ATP has been regenerated from ADP with propionylphosphate/acetate kinase, but propionylphosphate is a poorer substrate than AcP[18].

Carbamoyl phosphate (CP; 4)/carbamyl kinase (CK; E.C. 2.7.2.2) was described as a regeneration method for ATP in 1973 but it has seldom been used[70]. CP is a very strong phosphorylating agent (see Table 13-2) and can easily be prepared from aqueous potassium cyanate and KH_2PO_4[52]. Rapid decomposition of carbamyl phosphate generates ammonium ions and magnesium ammonium phosphate complex is formed as a gelatinous precipitate. This precipitation lowers activity, both by precipitating the magnesium(II) required for activity of the kinases, and because it occludes enzymes.

ADP phosphorylation with polyphosphate (P_n) and polyphosphate kinase (P_nK; E.C. 2.7.4.1) has also been demonstrated[44]. The cheap, stable polyphosphates and the stability of P_nK are highly attractive. Unfortunately, P_n has a low phosphorylating potential and P_nK is not readily available. These facts may explain why this regeneration system has not found any practical application.

Another very interesting but little-used regeneration method is based on phosphocreatine (PC; 7) and creatine kinase (CrK; E.C. 2.7.3.2)[71]. PC is comparable in its phosphorylating potential to AcP, but it is more stable in aqueous solutions (Table 13-3). CrK is inexpensive and fairly stable. The current lack of an efficient and simple laboratory scale synthesis for PC seems to have limited the applications of this method to a few syntheses of sugars[71] and nucleosides[72].

Recently, a promising regeneration system based on a multienzyme system from the glycolytic pathway was described[45] (Fig. 13-4). Glucose and inorganic phosphate were used as low-energy phosphorylating agents. Eleven enzymes are used to convert glucose to lactate. Two equivalents of inorganic phosphate are consumed and two equivalents of ATP are formed. The overall process of this regeneration system has a favorable free energy (see Table 13-3), which can be useful in the synthesis of compounds with high phosphorylating potentials (see Table 13-4, entry 20). The main backdraw of this method is the complexity of this multienzyme system and the poor stability of some of the enzymes used.

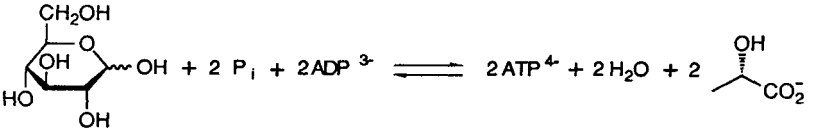


Figure 13-4. ATP regeneration via the glycolytic pathway[45]. Eleven enzymes are used to catalyze the conversion of glucose to lactate.

b) From AMP. In biochemical processes, ATP may be converted to either ADP or AMP. The regeneration of ATP from AMP is slightly more complicated than from ADP. Methods coupling one of the above mentioned regeneration systems and adenylate kinase (AdK; E.C. 2.7.4.3) have been used extensively in the production of ATP[48, 43]. AdK catalyzes the formation of 2 ADP from ATP and AMP. AcP/AcK and PEP/PK are the methods most often used to convert ADP to ATP (vide infra).

13.2.1.2
Regeneration of Other Nucleoside Triphosphates

The preparation of nucleoside triphosphates (NTP) from nucleoside diphosphates (NDP) follows the same regeneration systems described above (Section 13.2.1.1). AcK and PK have broad substrate specificities and recognize all of the NDPs[48, 73]. The efficient generation of NDP from nucleoside monophosphates (NMP) has been solved on preparative scale[74]. Adenylate kinase was used in the preparation of cytidine 5'-triphosphate (CTP) and uridine 5'-triphosphate (UTP) from the corresponding nucleoside monophosphates. Nucleoside monophosphate kinase (NMP; E.C. 2.7.1.4) was used in the synthesis of UTP. Guanosine 5'-triphosphate (GTP) was prepared with guanylate kinase as catalyst, coupled to a conventional ATP regeneration system. Best results were achieved when 3-phosphoglyceric acid served as the ultimate phosphorylating agent, and a multienzyme system was used as transfer catalyst (Fig. 13-5).

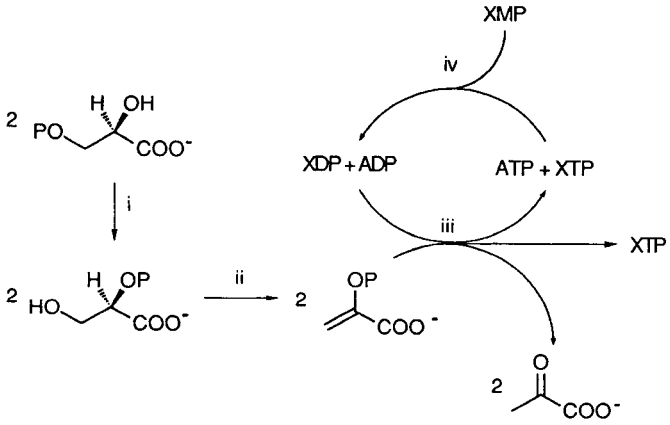


Figure 13-5. Enzymatic synthesis of nucleoside triphosphates. i) Phosphoglycerate mutase (E.C. 2.7.5.3); ii) enolase (E.C. 4.2.1.11); iii) pyruvate kinase (E.C. 2.7.1.40); iv) adenylate kinase (E.C. 2.7.4.3, X = A, C, U), guanylate kinase (E.C. 2.7.4.8, X = G) or nucleoside monophosphate kinase (E.C. 2.7.4.4, X = U). P = phosphate. See ref. [74].

13.2.2 Applications

13.2.2.1 Phosphorylations with ATP as a Cofactor

The most widely used and best developed enzyme-catalyzed phosphorylations are the ones that are coupled to ATP regeneration systems. Sugar phosphates, nucleoside phosphates and glycerides are the major classes of compounds prepared with these methods.

Kinases are the enzymes most often used for the phosphorylation of saccharides (Table 13-4, entries 7-19). For example, glucose-6-phosphate (**11**), a useful reagent for the regeneration of nicotinamide cofactors (see Chapter 15), was prepared from glucose in a one-step reaction by phosphorylation with ATP [75]. ATP was regenerated with AcP/AcK and the phosphoryl transfer was catalyzed with hexokinase, (HK; E.C. 2.7.1.1), and enzyme with broad substrate specificity. Both enzymes were immobilized and reused after product isolation. Alternatively, fluorinated hexopyranose phosphates and glucose phosphate analogs, with sulfur or nitrogen in the ring, were prepared with PEP/PK and HK [76] (Table 13-4, entry 9). The synthesis of **11** starting from polysaccharides or from fructose 1,6-diphosphate [77] was demonstrated, but the former method is less convenient and the latter is more expensive than the procedure starting from D-glucose.

5-phospho-D-ribosyl α-1-pyrophosphate (PRPP; **13**) is a key intermediate in the biosynthesis of various nucleotides and other natural products. An interesting application of an ATP/AMP regeneration system is demonstrated in the synthesis of PRPP from D-ribose in a multienzyme reaction [35] (Table 13-4, entries 13 and 14). In the first step, ribose was phosphorylated with ATP using ribokinase (RK; E.C. 2.7.1.17) as catalyst. In the second step, PRPP-synthetase (E.C. 2.7.6.1) catalyzed the transfer of a pyrophosphate group from ATP to ribose 5-phosphate (Fig. 13-6).

The preparations of ATP, GTP, CTP and UTP have been discussed in Section 13.2.1. Kinases are the enzymes most popular for the synthesis and regeneration of nucleoside triphosphates from their mono- and diphosphates. Nucleoside phosphate analogs have been synthesized using the same enzymes. For example, ribavarin triphosphate (RTP; **14**), a compound with anti-viral properties, was prepared from ribavarin monophosphate with adenylate kinase (E.C. 2.7.4.3) and pyruvate kinase (E.C. 2.7.1.40) as catalysts with PEP as ultimate phosphorylating agent (Fig. 13-7) [48]. Here PEP/PK has proved to be more useful as regeneration system for ATP than AcP/AcK (see Section 13.2.1.1) in a typical example of a kinetically unfavored reaction. RMP is one of the rare unnatural substrates accepted by adenylate kinase. Other nucleotide analogs – for example ATP-α-S and ATP-γ-S – have also been synthesized using kinases (Table 13-5, entries 4 and 5) [78, 79].

Glycerol kinase (GK; E.C. 2.7.1.30) catalyzes the enantiospecific phosphorylation of glycerol to form sn-glycerol-3-phosphate, an important intermediate for the synthesis of phospholipids. The enzyme is inexpensive and stable when immobilized. Studies with enzymes from a variety of microbial sources have shown

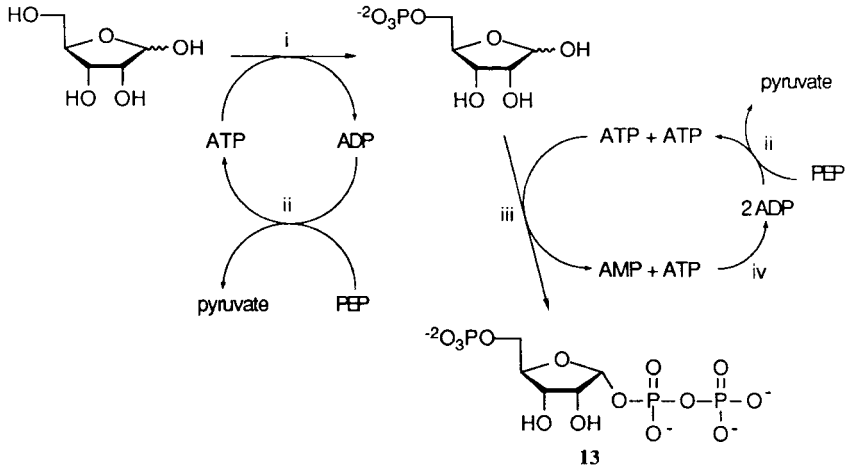


Figure 13-6. Coupled enzymatic synthesis of PRPP from D-ribose[35] i) ribokinase; ii) pyruvate kinase; iii) PRPP-synthetase; iv) adenylate kinase.

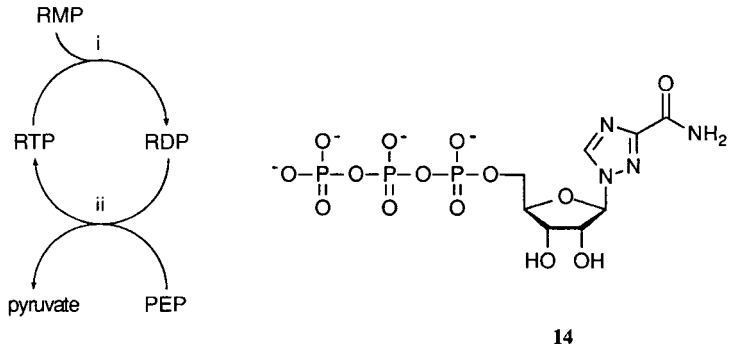


Figure 13-7. Enzymatic synthesis of ribavarin 5'-triphosphate (RTP; **14**) from ribavarin 5'-monophosphate (RMP)[48]. i) adenylate kinase (EC 2.7.4.3); ii) pyruvate kinase (EC 2.7.1.40).

that glycerol kinase accepts a wide range of glycerol analogs as substrate (Table 13-4, entries 3-5)[36]. sn-Glycerol-3-phosphate (**12**) and analogs were synthesized in gram scales, using glycerol kinase as catalyst and PEP/PK or AcP/AcK as ATP regeneration system. The phosphorylation of racemic mixtures produced chiral organic phosphates with enantiomeric excess (*ee*) >90–95% and yields of 75–95%. The unphosphorylated enantiomers of the chiral substrates were recovered in yields of 30–40% (80–90% *ee*). Alkaline phosphatase was used to hydrolyze the phosphorylated enantiomer and to provide enantiomerically pure unphosphorylated material[37]. For example, D-3-Chloropropane-1,2-diol (**15**) was prepared from a racemic mixture in a two step procedure with a 53% overall yield (97% *ee*) (Fig. 13-8).

Another application of glycerol kinase is the monophosphorylation of dihydroxy-

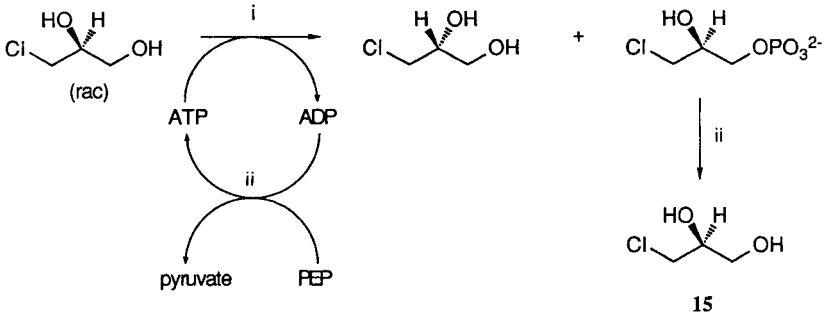


Figure 13-8. Enzyme catalyzed separation of a racemic mixture of D,L-3-chloropropane-1,2-diol. i) glycerol kinase; ii) alkaline phosphatase.

acetone[37]. Dihydroxyacetone phosphate (DHAP), an important intermediate in the aldolase catalyzed synthesis of monosaccharides (see Chapter 14), was prepared in a 0.4 mol scale using AcP/AcK for the regeneration of ATP (Table 13-4; entry 6). Guanidine derivatives were phosphorylated with ATP as well. The syntheses of arginine- and creatine phosphate (**7** and **9**), two relatively strong biological phosphorylating agents, are not economical, but they demonstrate the potentials of different ATP regeneration systems (see Table 13-4, entries 20 and 21). Further applications of ATP regeneration systems are given in Table 13-4 and 13-5.

13.2.2.2
P–O Bond Formation with Other Nucleoside Triphosphates than ATP

The use of stoichiometric quantities of nucleoside triphosphates or their regeneration from the corresponding mono- or diphosphates have found important applications in the synthesis of activated natural products; for instance, nucleoside phosphate sugars are important biological intermediates in the synthesis of complex carbohydrates, glycoproteins, glycolipids and proteoglycans. All of the eight nucleoside phosphate sugars, used *in vivo* by mammalian glycosyltransferases in the Leloir pathway, are accessible today by practical enzymatic or chemoenzymatic approaches (see reviews ref.[80] and [81]). The use of these activated monosaccharides in the enzymatic preparation of oligosaccharides and glycoconjugates is discussed in Section 11.3. The enzymatic synthesis of sucrose is, however, discussed here, to illustrate the efficient *in situ* generation of UDP-glucose from UDP in a complex multienzyme reaction (Fig. 13-9). In a typical example, a nucleoside triphosphate is recycled to regenerate a nucleoside phosphate sugar[82]. The synthesis, starting from glucose-1-phosphate and fructose, used sucrose synthetase (E.C. 2.4.1.13), pyruvate kinase (E.C. 2.7.1.40) and UDP-glucose pyrophosphorylase (E.C. 2.7.7.9). Inorganic pyrophosphatase (E.C. 2.6.1.1) was used to keep the pyrophosphate concentration in the reaction mixture low and to drive the equilibrium to the product side.

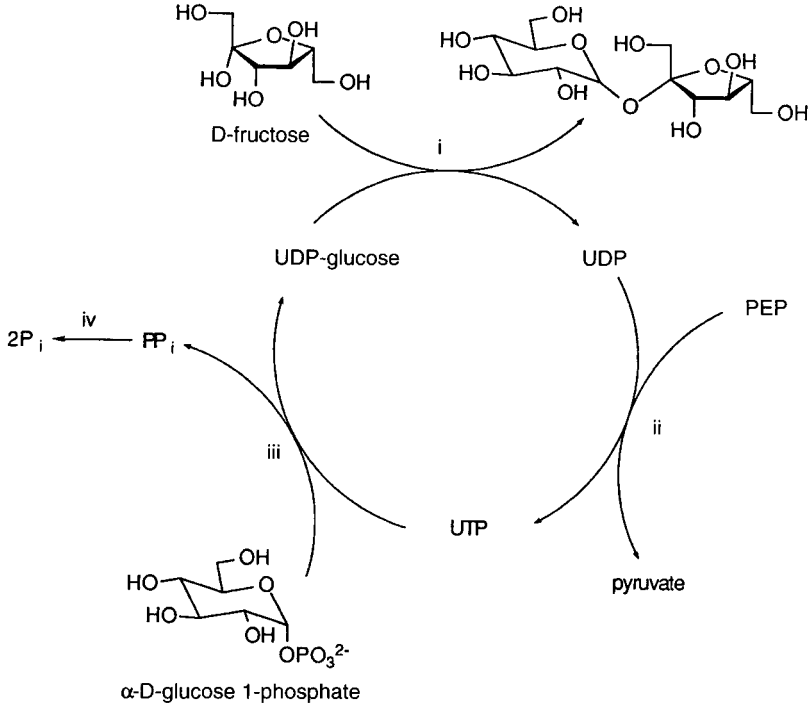


Figure 13-9. General scheme for an enzyme-catalyzed synthesis of sucrose with UDP-glucose[82]. i) sucrose synthetase; ii) pyruvate kinase; iii) UDP-glucose pyrophosphorylase; iv) inorganic pyrophosphatase.

13.2.2.3
Other Phosphorylating Agents

Agents other than ATP are rare in enzyme-catalyzed formation of P–O bonds. Inorganic phosphate, pyrophosphate and polyphosphates were used to prepare phosphorylated monosaccharides, alcohols, polyols[83] and phenols[84]. The yields were poor and the reactions lack specificity (Table 13-4, entries 1, 2 and 4). Glycerol-1-phosphate was prepared from glycerol, for instance, using inorganic phosphate and alkaline phosphatase (E.C. 3.1.3.1): 75 g of the product was isolated in a 41% yield[9]. The reaction was regio- but not stereospecific. Phosphorylases were used with isotopically labeled phosphate or with inorganic thiophosphate to prepare (thio)phosphorylated monosaccharides from oligo- or polysaccharides (see Table 13-4, entries 18 and 15). ^{32}P-labeling of phospholipase with [^{32}P]P_i was used to study the biological function of this enzyme complex[85]. *p*-Nitrophenyl phosphate was employed as phosphoryl group donor in the synthesis of allo-uridine, using a phosphotransferase as catalyst (see Table 13-5, entry 6).

Table 13-4. P–O bond formation at non-nucleoside compounds.

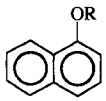
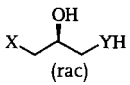
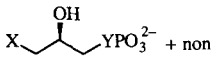
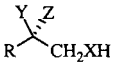
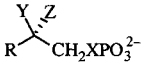
Entry	Starting material	Product	Enzyme	P-Source	Cofactor regeneration	References
Aliphatic and Aromatic Alcohols						
1	Monosaccharides, alcohols, polyols	Phosphorylated products	Alkaline Phosphatase (E.C. 3.1.3.1)	PP_i	none	[83]
2	 R = H	R = PO_3^{2-} (45%)	Alkaline Phosphatase (E.C. 3.1.3.1)	P_i		[84]
Glycerol, Glycerol Analogs, Dihydroxyacetone						
3	 (rac) X = Cl, SH, OCH_3, CH_2OH, Br, CH_2CH_3, OH; Y = O or NH	 + non phosphorylated enantiomer (29–95%)	Glycerol Kinase (E.C. 2.7.1.30)	ATP	PEP/PK or AcP/AcK	[37]; see also [43], [8], [83], [87], [9]
4	X = OH; Y = O	(rac)-glycerolphosphate	Alkaline Phosphatase (E.C. 3.1.3.1)	P_i, PP_i		[83], [9]
5	 X = O, NH, H; Y = OH, H, CH_2OH, F, SH, NH_2; Z = H, CH_3, CH_2CH_3; Y = Z = O.	 (45–96%)	Glycerol Kinase (E.C. 2.7.1.30)	ATP	PEP/PK	[36]; see also [88]

Table 13-4. (cont.).

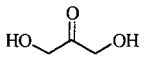
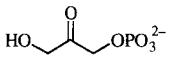
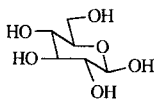
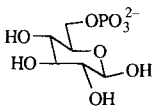
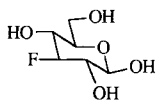
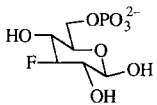
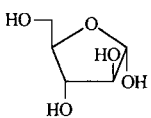
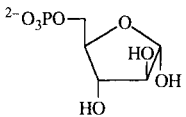
Entry	Starting material	Product	Enzyme	P-Source	Cofactor regeneration	References
6		 DHAP (83%)	GK (E.C. 2.7.1.30)	ATP	PEP/PK or AcP/AcK	[43]; see also [79]
Monosaccharides						
7		 **11** (65%)	Hexokinase (E.C. 2.7.1.1)	ATP	AcP/Ac	[75]
8	ibid	**11** (80%)	γ-Glutamyl-Cysteine Synthetase (E.C. 6.3.2.2)	ATP	AcP/AcK	[63]; see also [13], [89], [77]
9	 (and other glucose analogs)	 (86%)	Hexokinase (E.C. 2.7.1.1)	ATP	PEP/PK	[76]; see also [90]
10	 D-arabinose	 (63%)	Hexokinase (E.C. 2.7.1.1)	ATP	PEP/PK	[69]

Table 13-4. (cont.).

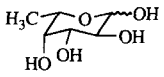
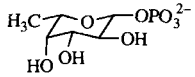
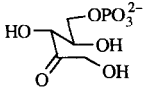
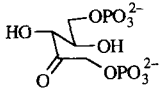
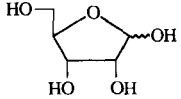
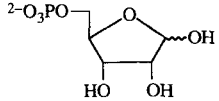
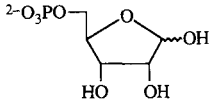
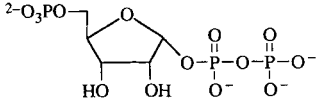
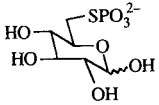
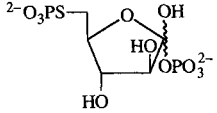
Entry	Starting material	Product	Enzyme	P-Source	Cofactor regeneration	References
11		 (80%)	Fucose Kinase (E.C. 2.7.1.52)	ATP	PEP/PK	[91]
12	 ribulose 5-phosphate	 ribulose 1,5-biphosphate	Phosphoribulokinase (E.C. 2.7.1.19)	ATP	PEP/PK (82%) or AcP/AcK (66%)	[35] [92]
13	 D-ribose	 D-ribose 5-phosphate (74%)	Ribokinase (E.C. 2.7.1.17)	ATP	PEP/PK	[35]
14	 D-ribose 5-phosphate	 PRPP (75%)	PRPP Synthase (E.C. 2.7.6.1)	ATP	AcP/AcK	[35]; see also [93], [94]
15	Glycogen	[^{35}P]glucose 1-phosphate	Phosphorylase a	[^{32}P]P_i	none	[95]
16			Fructokinase (E.C. 2.7.1.11) and Phosphoglucoisomerase (E.C. 5.3.1.9)	ATP	none	[96]

Table 13-4. (cont.).

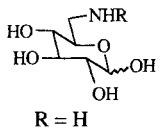
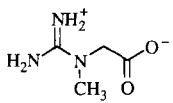
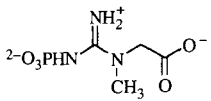
Entry	Starting material	Product	Enzyme	P-Source	Cofactor regeneration	References
17	Galactose	Galactose l-thiophosphate (25%)	Galactokinase (E.C. 2.7.1.6)	ATP(γS)	none	[97]
18	Sucrose	Glucose l-thiophosphate (55%)	Sucrose Phosphorylase (E.C. 2.4.1.7)	Na_3SPO_3	none	[97]; see also [98]
19	 R = H	R = PO_3^{2-} (18%)	Hexokinase (E.C. 2.7.1.1)	ATP	PEP/PK	[99]
Guanidine derivatives						
20	 creatine	 phosphocreatine	Creatine Kinase (E.C. 2.7.3.2)	ATP	AcP/AcK (24%)	[55]
					MCP/AcK (55%)	[18]
					Glucose, P_i + Mutienzyme system	[45]
21	L-Arginine	Phospho-arginine	Arginine Kinase (E.C. 2.7.3.3)	ATP	PEP/PK (67%) or AcP/AcK (31%)	[100]
Enzyme						
22	Phospholipase C	Phosphorylated Phospholipase	Acid Phosphatase (E.C. 3.1.3.2)	P_i		[85]

Table 13-5. P–O bond formation at nucleosides.

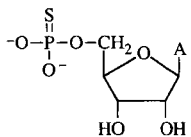
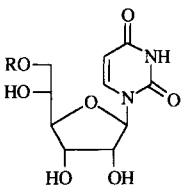
Entry	Starting Material	Product	Enzyme	P-Source	Cofactor regeneration	References
Nucleotides and Analogs						
1	dAMP (DNA)	dATP (67%)	Adenylate Kinase (E.C. 2.7.4.3)	ATP	PEP/PK	[94]
2	NMP (RNA)	ATP, GTP, CTP, UTP	NMP-Kinase (E.C. 2.7.4.4)	ATP	AcP/AcK	[40]; see also[101], [74]
3	RMP	RTP (**14**) (93%)	Adenylate Kinase (E.C. 2.7.4.3)	ATP	PEP/PK	[48]
4	 (AMPS)	ATP-α-S (53%)	AdK (E.C. 2.7.4.3)/PK (E.C. 2.7.1.40)	ATP	PEP/PK	[78]; see also[102]
5	ADP	ATP-γ-S (80%)	Phosphoglycerate-Kinase (E.C. 2.7.2.3)	1-(Thiophospho)-3-phospho-glycerate	Dihydroxyacetone, Na_2HSPO_3, Multienzyme System	[79]
6	 R = H (allo-uridine)	R = PO_3^{2-} (31%)	Phosphotransferase (Malt Sprouts)	*p*-Nitrophenyl phosphate	none	[103]; see also[104]

Table 13-5. (cont.).

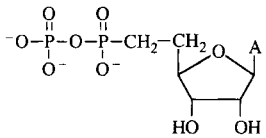
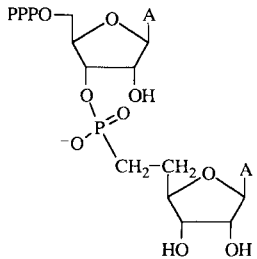
Entry	Starting Material	Product	Enzyme	P-Source	Cofactor regeneration	References
Oligonucleotides and Analogs						
7	Single Stranded DNA+ Mixture of Deoxynucleoside 5'-*O*-(1-thiotriphosphate) phosphorothioates	Phosphorothioate + Analogs of DNA	DNA Polymerase 1 (E.C. 2.7.7.7) and T_4 DNA Ligase (E.C. 6.5.1.1)			[105]
8	 A=Adenine	 (~90%)	Polynucleotide Phosphorylase (E.C. 2.7.7.8)			[106]
9	ATP	Ap_4A (98%)	Leucyl t-RNA Synthetase (E.C. 6.1.1.4)	ATP	AcP/AcK AdK	[12] see also[11, 13, 107]
Nucleoside Phosphate Sugars						
10	UTP + Glucose 1-Phosphate	UDP-Glucose (95%)	UDP-Glucose Pyrophosphorylase (E.C. 2.7.7.9)			[73] see also[86, 95]

Table 13-5. (cont.).

Entry	Starting Material	Product	Enzyme	P-Source	Cofactor regeneration	References
11	Galactose l-thiophosphate and UDP-glucose	Uridine-5'-*O*-(2-thiodiphosphogalactose) (UDP(ßS)-Galactose (50%)	Galactose-1-Phosphate Uridyl Transferase (E.C. 2.7.7.12)			[97]
NAD(P)$^+$						
12	ATP + Nicotinamide Mononucleotide	NAD^+ (> 90%)	NAD Pyrophosphorylase (E.C. 2.7.7.1)	ATP	AcP/AcK AdK	[108]
13	NAD^+	$NADP^+$ (quant)	NAD Kinase (E.C. 2.7.1.23)	ATP	AcP/AcK	[108]

13.2.3
Tables Containing Typical Examples Ordered According to the Classes of Compounds

Sugars, nucleosides and their analogs are the classes of compounds most often involved in enzyme catalyzed phosphorylation. Typical carbohydrate phosphorylations are included in Table 13-4, together with the phosphorylation of other non-nucleosidic compounds. Table 13-5 gives an overview of the enzyme catalyzed phosphorylation reactions of nucleosides and their analogs. A few representative examples of nucleoside sugars are listed, for more detailed information consult the review. refs [74, 80, 81].

13.3
Cleavage of P–O Bonds

In vivo, cleavage of P–O bonds are performed by enzymes such as phosphatases, phosphodiesterases, phosphohydrolases, nucleases, DNases and RNases (see Section 13.1.1). *In vitro*, cleavage of a P–O bond is often a trivial synthetic step. Even for an easy step, enzymes attract increasing attention. The enzymatic reactions are preferred when regio- or stereoselectivity is required, and when the substrates are temperature or pH sensitive. Many phosphate analogs have been tested as substrates of enzymes that hydrolyze phosphoryl groups. These analogs are often accepted as substrates for the enzymes, and such reactions could be synthetically valuable. Typical examples are presented in the tables.

Table 13-6. Hydrolysis of phosphate and pyrophosphate monoester.

$R{-}O{-}PO_3^{2-} \longrightarrow R{-}OH$
$R{-}O{-}P(O_2^-){-}PO_3^{2-} \longrightarrow R{-}OH$

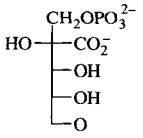
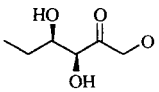
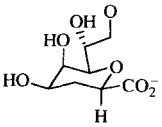
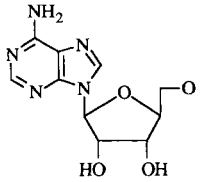
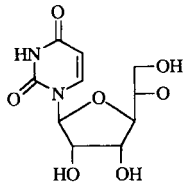
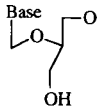
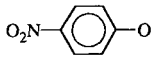
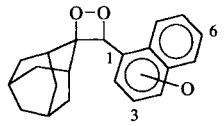
Entry	R–O	Enzyme	References
1	Polyprenol (phosphates and pyrophosphates)	Acid Phosphatase (E.C. 3.1.3.2) or Alkaline Phosphatase (E.C. 3.1.3.1)	[110–116]
2		Acid Phosphatase (E.C. 3.1.3.2) or Alkaline Phosphatase (E.C. 3.1.3.1)	[117]
3		Acid Phosphatase (E.C. 3.1.3.2)	[118]; see also[119–122]
4		KDO 8-Phosphate Phosphatase	[123]

Table 13-6. (cont.).

Entry	R–O	Enzyme	References
5		5'-Ribonucleotide phosphohydrolase (E.C. 3.1.3.5)	[124]
6		Alkaline Phosphatase (E.C. 3.1.3.1) or Acid Phosphatase (E.C. 3.1.3.2)	[103]
7		Alkaline Phosphatase (E.C. 3.1.3.1)	[104]
8		Alkaline Phosphatase (E.C. 3.1.3.1) or Acid Phosphatase (2-Phases System) (E.C. 3.1.3.2)	[125, 126]
9	 O in 3 or 6	Alkaline Phosphatase (E.C. 3.1.3.1)	[127]
10		Inorganic Pyrophosphatase (E.C. 3.6.1.1)	[35]

13.3.1
Hydrolysis of Phosphate and Pyrophosphate Monoesters

Both acid and alkaline phosphatases have been used to cleave aliphatic and aromatic phosphate monoesters. Table 13-6 shows typical examples ordered according to the substrate class. This table includes an example where the enzymatic reaction was run with a sensitive substrate (entry 1), and examples where regio- or stereoselectivity was required (entries 2 and 5, respectively).

Polyprenyl phosphates and pyrophosphates have been hydrolyzed by acid and alkaline phosphatases (Table 13-6, entry 1). For this hydrolysis, classical chemical methods are inadequate as the reaction products decompose under acid conditions[109].

A regioselective dephosphorylation was used in the synthesis of 2'-carboxy-D-arabinitol 1-phosphate (Table 13–6, entry 2), a natural inhibitor of ribulose 1,5-bisphosphate carboxylase. Either acid or alkaline phosphatases can be used for the selective hydrolysis of the 1-phosphoryl group of 2'-carboxyl-D-arabinitol 1,5-bisphosphate. With acid phosphatase, the conversion was essentially quantitative yielding exclusively the 1-phosphate derivative (cleavage of the 5-phosphoryl group). On the other hand, hydrolysis with alkaline phosphatase gave a 4 : 1 mixture of the 1- and 5-phosphate derivatives.

Many natural and unnatural monosaccharides have been prepared by aldolase catalyzed condensation. The synthesized sugars were often dephosphorylated *in situ* by an acid phosphatase (Table 13-6, entry 3). These reactions illustrate multienzyme synthesis. In this case, no isolation of the phosphate intermediate is required: both enzymatic reactions are run in the same pot after adjustment of the pH value.

One of the best examples of an enzymatic dephosphorylation for a synthetic purpose is shown in the entry 5 of Table 13-6. A 5'-ribonucleotide phosphohydrolase was used in the synthesis of (–)-aristeromycin, a carbocyclic analog of adenosine. The (–)-enantiomer of aristeromycin shows some cytostatic and antiviral activity, while the (+)-enantiomer is inactive. The racemate (±)-5'-phosphorylated aristeromycin was resolved by selective hydrolysis of the (–)-enantiomer with the hydrolase. The (–)-alcohol and the (+)-5'-phosphate derivative were separated easily on a silica gel column. Hydrolysis of the (+)-enantiomer with calf intestinal phosphatase yielded pure (+)-alcohol.

Phosphorylated *p*-nitrophenol was hydrolyzed with an alkaline phosphatase[129]. This hydrolysis was also performed in a two-phase system with an acid phosphatase[125].

The naphtol derivative, Table 13-6, entry 9, is dephosphorylated by an alkaline phosphatase. The resulting naphtol decomposes with chemiluminescent emission and can be used in bioassays to generate a chemiluminescence signal proportional to the concentration of an alkaline phosphatase label.

Inorganic pyrophosphate may be considered as a particular case of a phosphate monoester. The enzymatic decomposition of pyrophosphate by inorganic pyrophosphatase (Table 13-6, entry 10) can be used to drive a multienzyme synthesis (see[35]).

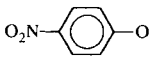
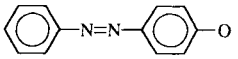
13.3.2
Hydrolysis of *S*- and *N*-substituted Phosphate Monoester Analogs

Enzymatic hydrolysis of oligonucleotide-analogs containing modified phosphoryl moieties have been examined extensively to study their resistance to the enzymatic hydrolysis.

Thiophosphates (Table 13-7) were subjected to hydrolysis with both acid and alkaline phosphatases. Most authors claimed that these compounds are substrates for alkaline phosphatases, but the reaction rate is much lower than with the corresponding phosphates[128, 126]. Neumann[129], however, reported that these same *S*-substituted analogs are resistant to alkaline phosphatases but hydrolyzed by acid phosphatases.

Table 13-7. Hydrolysis of thiophosphates.

$R–O–PSO_2^{2-} \longrightarrow R–OH$

Entry	R–O	Enzyme	References
1	$H_3C–O$	Alkaline Phosphatase (E.C. 3.1.3.1)	[128, 129]
2		Alkaline Phosphatase (E.C. 3.1.3.1) or Acid Phosphatase (E.C. 3.1.3.2)	[126, 129]
3		Alkaline Phosphatase (E.C. 3.1.3.1)	[128]

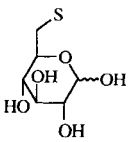
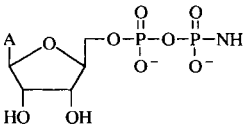
Only the alkaline phoshatases have been used with phosphorothioates (Table 13-8). The presence of sulfur between the phosphoryl moiety and the residue does affect the enzymatic reaction with alkaline phosphatases.

Imidodiphosphates are also potential substrates for phosphoryl hydrolyzing enzymes (see Table 13-8, entry 7). They have been used less often than the *S*-substituted phosphate analogs.

Another goal of these studies involving analogs with modified phosphoryl groups or isotopically labeled nucleotides was mechanistic elucidation of the stereochemical course of the reaction[15, 132, 133].

Table 13-8. Hydrolysis of phosphorothioates and imidodiphosphates.

$R–S–PO_3^{2-} \longrightarrow R–SH$
$R–NH–PO_3^{2-} \longrightarrow R–NH_2$

Entry	R–S or R–NH	Enzyme	References
1	$H_2N–CH_2–CH_2–S$	Alkaline Phosphatase (E.C. 3.1.3.1)	[129]
2	$H_3C–\overset{O}{\overset{\|\|}{C}}–NH–CH_2–CH_2–S$	Alkaline Phosphatase (E.C. 3.1.3.1)	[129]
3	$^-OOC–CH_2–CH_2–S$	Alkaline Phosphatase (E.C. 3.1.3.1)	[129]
4		Alkaline Phosphatase (E.C. 3.1.3.1)	[130]
5		Alkaline Phosphatase (E.C. 3.1.3.1) or Pyruvate Kinase (E.C. 2.7.1.40)	[130]
6		Alkaline Phosphatase (E.C. 3.1.3.1)	[96]
7		Alkaline Phosphatase (E.C. 3.1.3.1)	[131]

13.3.3
Hydrolysis of Phosphate and Phosphonate Diesters

13.3.3.1
Nucleic Acids and their Analogs

Endo- and exonucleases have been used successfully with nucleic acids and their analogs for organic synthetic purposes. For example, ATP was synthesized from AMP for use in cofactor recycling (Table 13-9, entry 1). The AMP was obtained from yeast RNA by cleavage with the nuclease P_1 yielding a mixture of nucleoside monophosphates[101]. In another report[73], nucleoside diphosphates were obtained by hydrolysis of RNA with nuclease P_1 and a polynucleotide phosphorylase (the diphosphates are preferred because the diphosphates were more easily transformed to the nucleoside triphosphates than the monophosphates).

Similarly, dATP was synthesized from dAMP, obtained by cleaving herring sperm DNA with DNase I and nuclease P_1 (Table 13-9, entry 2). Selective phosphorylation was obtained with adenylate kinase in the presence of pyruvate kinase and phosphoenol pyruvate.

Synthetic oligonucleotide analogs are interesting in applications in which they suppress translation of mRNAs by hybridization (antisense technology). A good antisense agent would be resistant to nucleases, and able to maintain its biological activity for substantial periods in living organisms[135]. Oligonucleotide analogs modified at the phosphodiester linkage with a phosphorothioate group are the subject of numerous papers (see Table 13-9). Other oligonucleotide analogs have been tested as substrates for endo- and exonucleases. The natural substrates were modified at either the base residues (Table 13-9, entry 4) or at the sugar moieties (Table 13-9, entries 5, 6 and 7).

The tetraphosphate Ap_4A and its analogs are other examples of a cleavage of a phosphodiester (Table 13-9, entry 8).

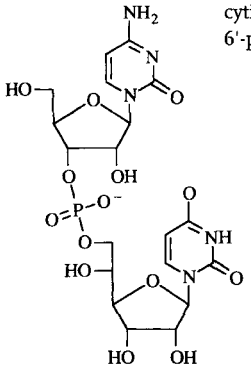
13.3.3.2
Other Phosphate and Phosphonate Diesters

Enzymes have often been used as mild catalysts to hydrolyze phosphate and phosphonate diesters.

Cyclic phosphate diesters can be hydrolyzed selectively with RNases and phosphodiesterases to give the corresponding phosphate monoesters (Table 13-10, entries 1 and 2).

Phosphodiesterases have been used to deprotect phosphonate diesters (Table 13-10, entries 3-5). This method is especially useful for sensitive compounds (see Table 13-10, entry 6: a P–O bond could be cleaved selectively in the presence of a P–N bond).

Table 13-9. Hydrolysis of nucleic acids, nucleosides and their analogs.

Entry	Starting material	Product	Enzyme	References
1	RNA	Nucleoside Monophosphates or Nucleoside Diphosphates	Nuclease P_1 (E.C. 3.1.30.1) or Nuclease P_1 (E.C. 3.1.30.1) and Polynucleotide Phosphorylase (E.C. 2.7.7.8)	[101, 73]
2	denatured DNA	Deoxy Nucleoside Monophosphates	DNase I (E.C. 3.1.21.1) Nuclease P_1 (E.C. 3.1.30.1)	[94]
3	Phosphorothioate Substituted Nucleic Acids		Endo- (E.C. 3.1.30.1) and Exonucleases (E.C. 3.1.4.1) (E.C. 3.1.16.1)	[105, 134–140]
4	Nucleotide Analogs Containing Modified Bases		Restriction Endonucleases	[141]
5	Nucleic Acid Analogs Containing L-Ribose		Exonucleases	[142]
6		cytidine + allo-uridine 6'-phosphate	RNase A (E.C. 3.1.27.5) RNase T_2 (E.C. 3.1.27.1) Nuclease S_1 (E.C. 3.1.30.1)	[103]
7	Nucleotide Analogs Containing Acyclic Sugar Analogs		Nucleases Phosphodiesterases (E.C. 3.1.16.1)	[143, 144]
8	Thiophosphate Analogs of AppppA		Ap_4 Hydrolases (E.C. 3.6.1.17) (E.C. 3.6.1.41) Ap_4A Phosphorylase (E.C. 2.7.7.53)	[145, 107]

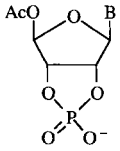
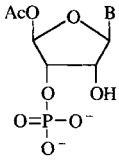
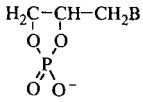
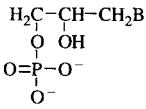
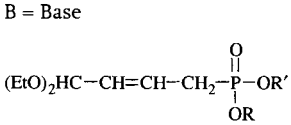
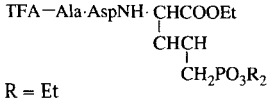
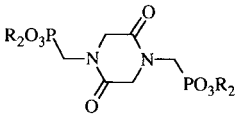
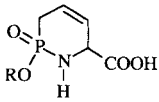
13.3.4
Other P–O Bond Cleavages

Phosphate and phosphonate esters can also be cleaved enzymatically to give products different from those obtained by enzymatic hydrolysis.

The formal migration of a phosphoryl group between the C_6 and the C_1 of glucose is catalyzed by phosphoglucomutase. Mechanistic studies were performed with the

Table 13-10. Hydrolysis of phosphate and phosphonate diesters.

$R–O–PO_3R'_2 \longrightarrow R–O–PO_3HR'$
$R–CR'_2–PO_3R''_2 \longrightarrow R–CR'_2–PO_3H_2$ or $R–CR'_2–PO_3HR''$

Entry	Starting Material	Product	Enzyme	References
1			RNase T_1 (E.C. 3.1.27.3) and RNase T_2 (E.C. 3.1.27.1)	[146]
2	 B = Base		RNases or Phosphodiesterase	[147]
3	 R = R′ = Et	R = R′ = H	Phosphodiesterase I (E.C. 3.1.4.1)	[148, 149]
4	 R = Et	R = H	Phosphodiesterase I (E.C. 3.1.4.1)	[148]
5	 R = Et	R = H	Phosphodiesterase I (E.C. 3.1.4.1)	[150]
6	 R = Et	R = H	Phosphodiesterase I (E.C. 3.1.4.1)	[148]

thiophosphate analog of glucose 6-phosphate **16**. In the presence of phosphoglucomutase, this analog yields 6-thioglucose 1-phosphate **17**; albeit at a slower rate than the natural substrate (Figs. 13-10).

Aminolysis of phosphonate diester derivatives have been used to form organophosphorus analogs of peptides (**18**) with phosphatases and phosphodiesterases[151, 152].

The equilibrium between phosphoenolpyruvate and phosphonopyruvate (**19**, Fig. 13-10) is catalyzed by a phosphomutase. The mechanism of the transformation of a phosphoryl into a phosphonoyl group has been studied with labeled and *S*-substituted analogs of the natural substrate[153–158].

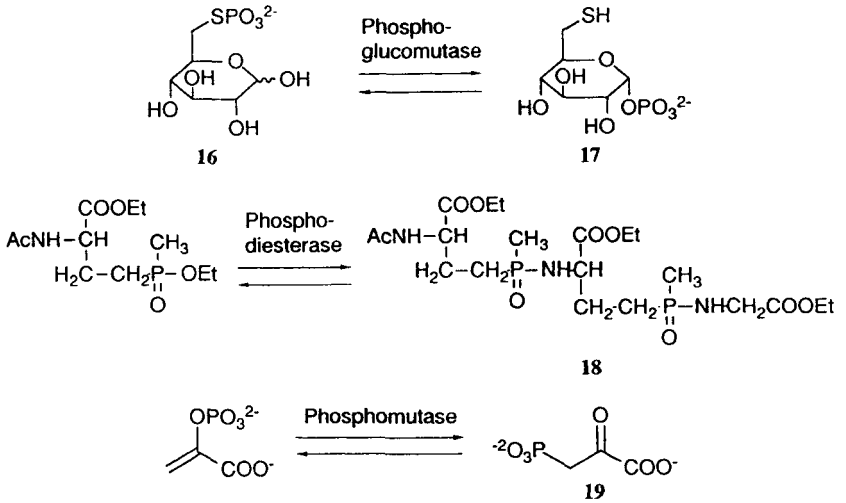


Figure 13-10. P–O bond cleavages with hydrolytic enzymes, not leading to the products of hydrolysis.

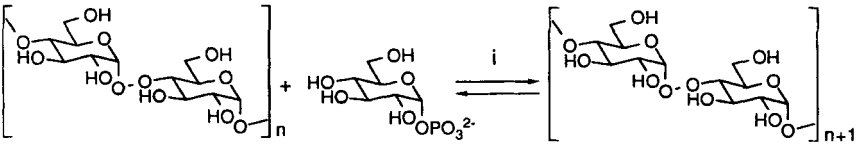


Figure 13-11. Phosphorylase catalyzed formation of polysaccharides and modified polysaccharides. i) phosphorylase.

Numerous analogs of carbohydrate polymers (i.e., amylose, glycogen) have been prepared from modified monosaccharide 1-phosphates with phosphorylase (Fig. 13-11 shows the natural substrates) [159–162].

Abbreviations

AcK: acetate kinase; AcP: acetyl phosphate; AdK: adenylate kinase; AP_nA: p^1,p^n-di(adenosine 5'-) *n*-phosphate; ARS: aminoacyl *t*RNA synthetase; ATP, ADP, AMP: adenosine 5'-tri-, di-, monophosphate; ATP-α-S: (S_p)-adenosine 5'-*O*-(1-thiotriphosphate), ATP-γ-S: adenosine 5'-*O*-(3-thiotriphosphate); CK: carbamyl kinase; CP: carbamyl phosphate; CrK: creatine kinase; CTP, CDP, CMP: cytidine 5'-tri-, di-, monophosphate; dATP, dAMP: deoxyadenosine 5'-tri-, monophosphate; DNA: deoxyribonucleic acid; ΔG: change in free energy; GK: glycerol kinase; GTP, GDP, GMP: guanosine 5'-tri-, di-, monophosphate; HK: hexokinase; IUB: International Union of Biochemistry; MCP: methoxycarbonyl phosphate; NTP, NDP, NMP: nucleoside 5'-tri-, di-, monophosphate; PC: phosphocreatine; PEP: phosphoenol pyruvate; P_i: orthophosphate; PK: pyruvate kinase; P_n: polyphosphate; P_nK: poly-

phosphate kinase; PP_i: pyrophosphate; PRPP: 5-phospho-D-ribosyl α-1-pyrophosphate; RNA: ribonucleic acid; *t*RNA: transfer RNA; RK: ribokinase; RTP, RMP: ribavarin tri-, monophosphate; U: one unit: the amount of enzyme that catalyzes the formation of 1 μmol/minute; UTP, UDP, UMP: uridine 5'-tri-, di-, monophosphate.

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Enzyme Catalysis in Organic Synthesis
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14
Formation of C-C Bonds

Chi-Huey Wong

14.1
Aldol Reactions

The aldol reaction is one of the most powerful methods for carbon-carbon bond formation, and its catalytic asymmetric variants have great potential in contemporary organic synthesis [1]. Aldolases are enzymes which catalyze reversible and irreversible asymmetric aldol condensations in nature [2–5], via one of two distinct reaction mechanisms [6]. Type I aldolases activate the donor/nucleophilic substrate via Schiff base formation with an active-site lysine residue. These enzymes are predominantly found in animals and higher plants, and do not require metal cofactors. Type II aldolases activate both donor and acceptor substrates via chelation to an active-site Zn^{2+}, and are found mainly in microorganisms. Aldolases can be conveniently classified into groups according to their natural donor substrates, i.e. dihydroxyacetone phosphate (DHAP), pyruvate/phosphoenol pyruvate (PEP), glycine, acetaldehyde, and a small number of other molecules. The ability of aldolases to accept a variety of unnatural acceptor substrates, and to generate new stereocenters of known absolute and relative stereochemistry reliably, has made them powerful tools for asymmetric synthesis.

14.1.1
DHAP-Utilizing Aldolases

14.1.1.1
Fructose 1,6-Diphosphate (FDP) Aldolase (E.C. 4.1.2.13)

FDP aldolase catalyzes the reversible aldol addition reaction of DHAP and D-glyceraldehyde 3-phosphate (D-Gly 3-P) to form D-FDP (Fig. 14.1-1). The equilibrium constant for this reaction has a value of $\sim 10^4$ M^{-1} in favor of FDP formation. The enzyme has been isolated from a variety of eukaryotic and prokaryotic sources, both in type I and type II forms [7–21]. Generally, the type I FDP aldolases exist as tetramers (M.W. ~ 160 KDa), while the type II enzymes are dimers (M.W. ~ 80 KDa). For the

Figure 14.1-1. Aldol addition reaction catalyzed *in vivo* by FDP aldolase.

type I enzymes there is a high degree of sequence homology (<50%), with the active site residues being highly conserved through evolution[12–22]. However, significant differences identified in the *C*-terminal regions may control substrate specificity[22]. No sequence homology between type I and type II aldolases, or between different type II enzymes, has been identified. Mechanistic studies have mainly been carried out on FDP aldolases from rabbit muscle (RAMA)[23] and yeast[24], and the X-ray structures of the enzymes from rabbit muscle (2.7 Å resolution)[22] and human muscle (3 Å resolution)[25] have been determined. Some of the type I aldolases are commercially available, inexpensive, and have useful specific activity (~60 U mg^{-1}). These enzymes are not particularly air-sensitive, though there is an active site thiol group. The free enzyme has a half-life of ~2 days in aqueous solution at pH 7.0[26, 27], but this is lengthened by immobilization or enclosure in a dialysis membrane. The type I aldolase from rabbit muscle has been cloned and expressed in *E. coli*[8]. The equivalent enzyme from *Staphylococcus carnosus* is much more stable for synthesis purposes[28]. The type II aldolases from several microbial sources have recently been cloned and overexpressed[12, 27, 29, 30]. Despite the small degree of homology in primary sequence between the enzymes from *E. coli* and rabbit muscle, studies have shown that they possess almost the same substrate specificity[31].

To date, FDP aldolase, especially the commercially available RAMA, is the most widely-used aldolase in organic synthesis. A few studies which compare the stability and kinetic parameters of RAMA vs. bacterial fructose-1,6-bisphosphate aldolases have been reported[32, 33], and FDP aldolase from spinach leaves has also been employed for synthesis purposes. RAMA accepts a wide range of aldehyde acceptor substrates, with DHAP as the donor, to generate vicinal diols with D-*threo* stereochemistry reliably[5, 26, 27, 35–44]. Suitable acceptors include unhindered aliphatic and α-heteroatom substituted aldehydes[26], monosaccharides, and derivatives thereof[44]. Aromatic, sterically hindered aliphatic, and α, β-unsaturated aldehydes are generally not substrates[26]. The specificity for the donor substrate is much more stringent. Initially, only three DHAP analogues were shown to be substrates, but they were so weak (~10% cf. DHAP), that their general use in organic synthesis was precluded[26, 45]. However, recently, a DHAP phosphonate analog has been shown to be a good substrate for FDP aldolases from rabbit and *S. carnosus*, as well as Rha 1-P aldolase from *E. coli*[32].

FDP aldolase exhibits kinetic diastereoselectivity with unnatural chiral aldehyde acceptor substrates. However, even though there is significant discrimination (~20 : 1) between the D- and L-enantiomers of the natural substrate Gly 3-P[26], this is usually not the case with unnatural aldehydes. In fact, resolutions of racemic aldehydes are normally only successful if carried out under thermodynamic control. Often the aldol products can cyclize via formation of a hemiketal, leading to

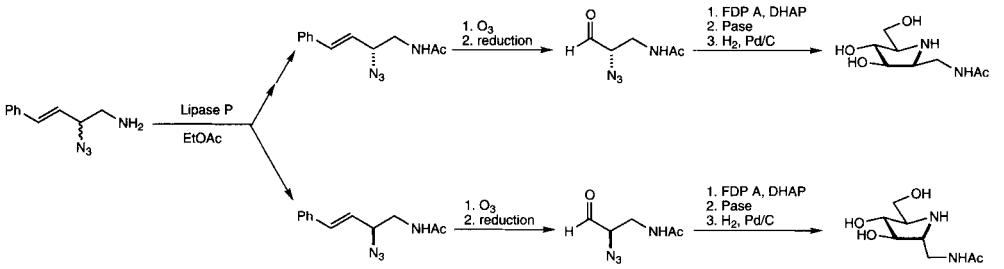


Figure 14.1-2. Preparation of optically active aldehyde acceptors for FDP aldolase.

significant energy differences between the two diastereomeric products, and ultimately favoring one product after equilibration. For example, with racemic β-hydroxybutyraldehyde[26, 37] as a substrate, only a single diastereomer was obtained, with the methyl group in the more stable equatorial position.

Synthetically, FDP aldolase has been employed in the production of ^{13}C-labeled[35, 46, 47], nitrogen-containing[27, 38–40], deoxy-[35–37], fluoro-[36, 48], and high-carbon sugars[34, 35, 37, 43]. Most of these syntheses require the preparation of the aldehyde acceptor. In cases where the aldehyde is optically active, this necessitates either asymmetric synthesis of the required enantiomer, or use of a racemic aldehyde, with subsequent separation of diastereomeric products. In general, ozonolysis of a terminal olefin (Fig. 14.1-2)[45] and acid-catalyzed acetal deprotection are convenient routes to the acceptor aldehydes. α-Chiral aldehydes have also been prepared by ring opening of readily-available (*R*)- and (*S*)-glycidaldehyde acetal, or the corresponding thirane and aziridine, by appropriate nucleophiles[49]. Both enantiomers of glycidaldehyde acetal may be prepared by lipase-catalyzed resolution of 3-chloro-2-hydroxypropanal diethyl acetal[49]. Alternatively, tandem use of Sharpless asymmetric dihydroxylation (AD) and aldolase-catalyzed condensation allows quick and facile synthesis of carbohydrates with complete stereocontrol (Fig. 14.1-3)[50].

A 1 : 4 mixture of deoxynojirimycin and deoxymannojirimycin was obtained when racemic 3-azido-2-hydroxypropanal was used as a substrate for RAMA[38, 39], indicating that the D-aldehyde is a better substrate for the enzyme. A similar result was obtained with FDP aldolase from *E. coli*[27]. Since both deoxynojirimycin and

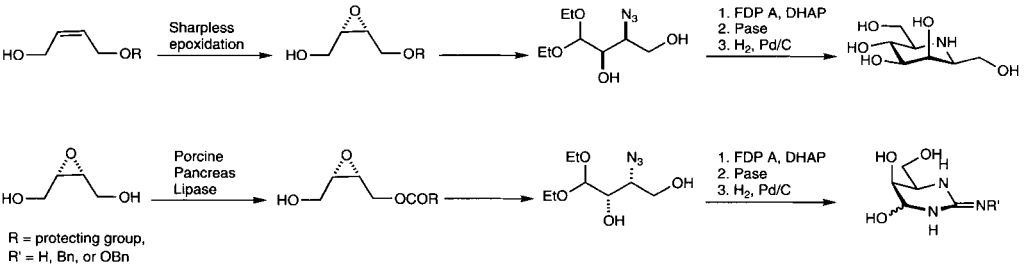


Figure 14.1-3. Chemoenzymatic stereo-controlled synthesis of azasugars.

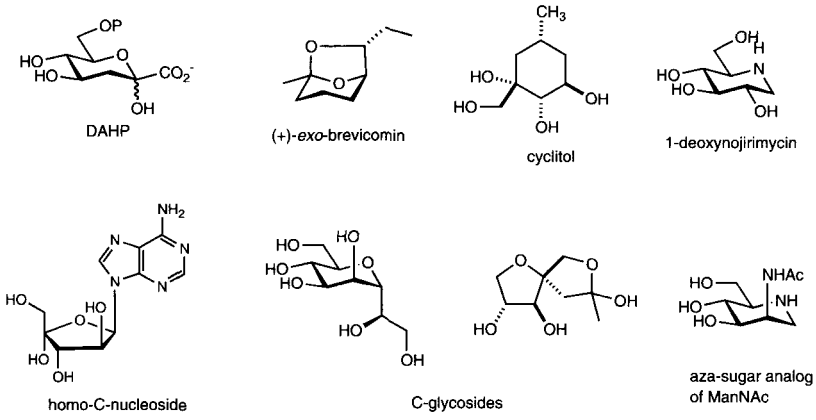


Figure 14.1-4. Various classes of molecules synthesized using FDP aldolase.

deoxymannojirimycin are potent glycosidase inhibitors, each compound was also prepared in an optically pure form from the respective optically pure azidoaldehydes[27]. Both (*R*)- and (*S*)-azido-2-hydroxypropanal were obtained via LP-80 catalyzed resolution of the racemic acetal precursor[49]. Similar strategies have been employed to prepare the β-glycosidase inhibitors β-1-homonojirimycin, β-1-homomannojirimycin[51], and the azasugars corresponding to *N*-acetylglucosamine and *N*-acetylmannosamine (Fig. 14.1-4)[49].

Similarly, employing 2-azidoaldehydes as RAMA substrates allowed the preparation of polyhydroxylated pyrrolidines (Fig. 14.1-5)[38, 52, 53]. 1,4-Dideoxy-1,4-imino-D-arabinitol[52] was synthesized from azidoacetaldehyde, and both (2*R*,5*R*)-[49] and (2*S*,5*R*)-bis(hydroxymethyl)-(3*R*,4*R*)-dihydroxypyrrolidine[53] were synthesized from racemic 2-azido-3-hydroxypropanal, respectively. In the latter case, the kinetic product of the aldol addition was transformed into the (2*R*,5*R*)-stereoisomer of the pyrrolidine, while the thermodynamic product gave the (2*S*,5*R*)-stereoisomer. Furthermore, pyrrolidines structurally related to GlcNAc have been prepared stereoselectively by a similar transformation from lipase-resolved aldehyde precursors[54].

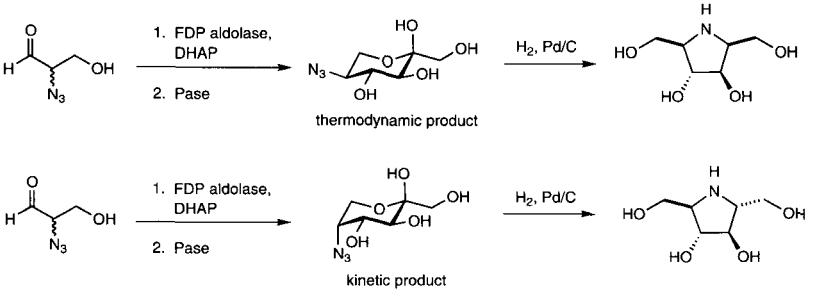


Figure 14.1-5. Synthesis of polyhydroxylated pyrrolidines using RAMA.

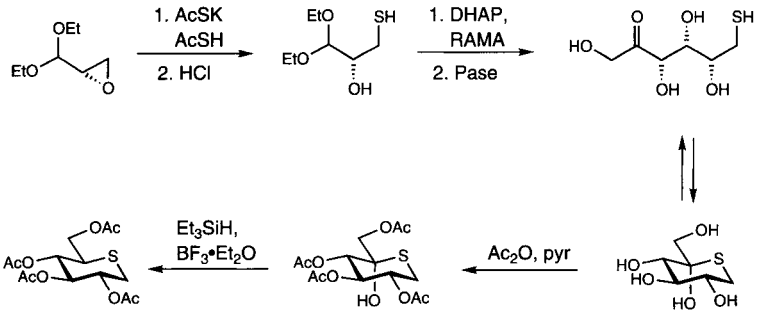


Figure 14.1-6. Preparation of deoxy-thio sugars.

The 6-deoxyazasugars and their analogs can also be easily prepared by direct reductive amination of the aldol products prior to removal of the phosphate group[55]. Studies using glucose 6-phosphate (Glc 6-P) indicate that the phosphate group is probably reductively cleaved from the imine 6-phosphate rather than the azasugar 6-phosphate. Use of 3-azido-4-hydroxy aldehydes results in the formation of homoaza sugars[51, 56]. The optically pure aldehydes can be obtained either by Sharpless epoxidation of the olefins[51] or enzymatic resolution of the epoxides[57]. The lipase-resolved material was also used to prepare another class of glycosyl cation mimics, the tetrahydropyrimidines[58, 59]. These compounds exist in equilibrium with their guanadinotetraose forms which predominate at low pH. The tetrahydropyrimidines are potent inhibitors of α-galactosidase, due to their close resemblance to the transition state half-chair conformation of the enzymatic reaction. Interestingly, an inhibitor with an OBn group attached to the nitrogen has a much lower pK_a and inhibits α-galactosidase in the region of a physiological pH[59].

Similar to the synthesis of azasugars, a series of deoxy-thio sugars was prepared by aldol condensation of thioaldehydes with DHAP followed by reduction of the resulting thioketoses (Fig. 14.1-6)[60]. Regioselective ring opening of the (*S*)-glycidaldehyde diethyl acetal with potassium thioacetate introduced the thio function. RAMA-catalyzed aldol condensation followed by dephosphorylation gave the corresponding thioketose[61], which was then acetylated and reduced to the 1-deoxy-5-thio-D-glucopyranose peracetate[60]. Also, in a similar manner, 1-deoxy-5-thio-D-mannopyranose was obtained from the other aldehyde enantiomer, while a Fuc-1-P aldolase-catalyzed reaction provided 1-deoxy-5-thio-galactopyranose and 1-deoxy-5-thio-altropyranose, and Rha-1-P aldolase catalyzed reaction produced 1-deoxy-5-thio-L-mannopyranose[60].

With other aldolases in place of FDP aldolase, a wide range of other polyhydroxylated piperidines and pyrrolidines have been synthesized (*vide infra*)[53]. Aldolase-catalyzed condensation followed by reductive amination has become a general strategy for the synthesis of 5-[62], 6-[62, 63], and 7-membered[64] cyclic imine sugars. The resulting compounds have become the gold standard template for glycosidase inhibitor design.

Use of racemic methyl *N*-acetylaspartate β-semialdehyde as a substrate for RAMA provides a precursor to 3-deoxy-D-arabino-heptulosonic acid 7-phosphate (DAHP,

Fig. 14.1-4)[41]. This compound is an important intermediate in the shikimate pathway for the biosynthesis of aromatic amino acids in plants. The RAMA reaction produced the desired D-*threo* stereochemistry, and chemical reduction of the keto group gave the desired (6*R*)-stereoisomer in 60% diastereomeric excess. Other analogs of DAHP are also potentially available by this route, due to the broad substrate specificity of RAMA.

The use of pentose and hexose phosphates as RAMA substrates provides a route to high-carbon sugars, including analogs of sialic acid and KDO[44, 65, 66]. Other carbohydrate derivatives prepared by RAMA include unsaturated C8-C9 sugars[67], phosphonic acid derivatives[68], fluorescently-labeled fructose derivatives[69], perfluoroalkylated sugars[70], and those protected by thioacetals[71]. Furthermore, the *S. carnosus* enzyme has been employed for the synthesis of bicyclic sugars[72] and disaccharide mimetics[73].

Complex xylulose structures can also be synthesized by RAMA[74]. Employing a one-pot, three-enzyme system with RAMA, triose phosphate isomerase, and 1-deoxy-D-xylulose-5-phosphate synthase, 1-deoxy-D-xylulose-5-phosphate could be obtained in 47% yield[75]. Furthermore, a four-enzyme, one-pot system employing FDP-aldolase from *S. carnosus* furnished 5-deoxy-5-ethyl-D-xylulose[76].

The synthesis of (+)-*exo*-brevicomin (Fig. 14.1-4) was the first example of the use of RAMA to synthesize a non-carbohydrate derivative[77]. RAMA was employed to catalyze the key aldol addition step, in which the two chiral centers of the target molecule were established. RAMA has also been employed for the synthesis of a key fragment of (+) aspicilin[78], and for that of acyclic polyols[79]. Single aldol condensation on remote dialdehydes has also been achieved[80].

Other molecules synthesized by FDP aldolase include *C*-glycosides[43, 82] and cyclitols (Fig. 14.1-4)[83]. Cyclitols are an interesting class of bio-active compounds, and the use of aldolases provides a chemo-enzymatic strategy towards their synthesis. An example is the synthesis of nitrocyclitols which was accomplished by an FDP aldolase catalyzed reaction with nitroaldehyde, followed by a non-enzymatic intramolecular nitro-aldol reaction (Fig. 14.1-7)[83a]. A one-pot synthesis of cyclitols has been reported, involving an FDP aldolase-catalyzed reaction between a phosphonate aldehyde and DHAP. The aldol product cyclized *in situ* via an intramolecular Horner-Wadsworth-Emmons olefination to give the polyhydroxylated cyclopentane

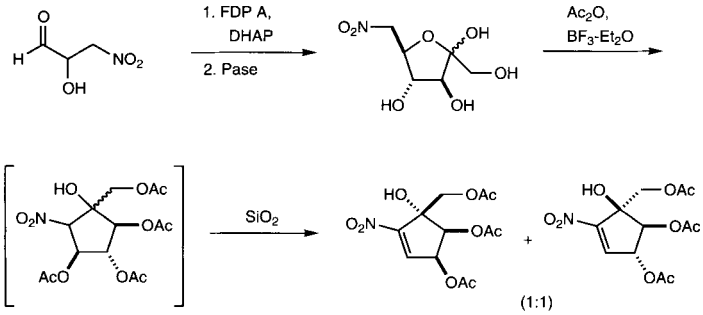


Figure 14.1-7. Preparation of nitro-cyclitols.

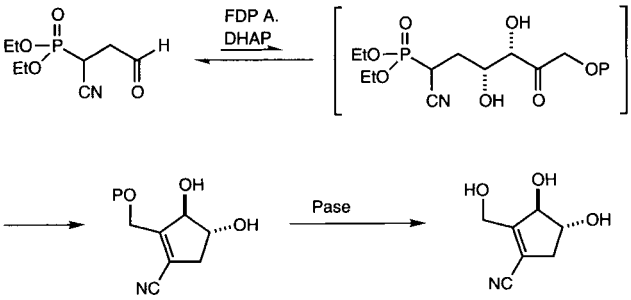


Figure 14.1-8. One-pot synthesis of cyclitols.

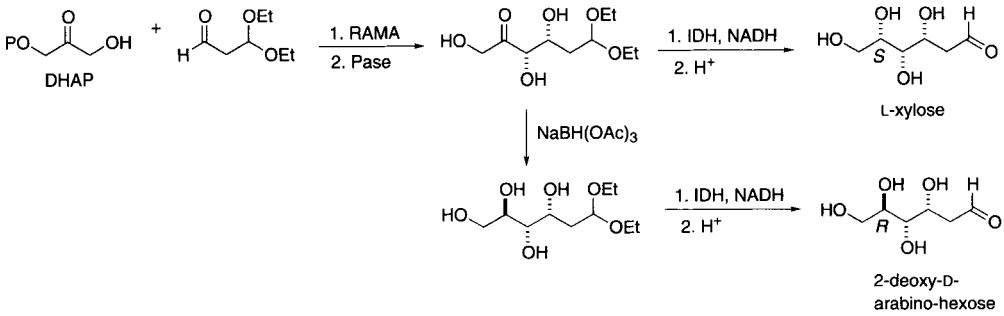
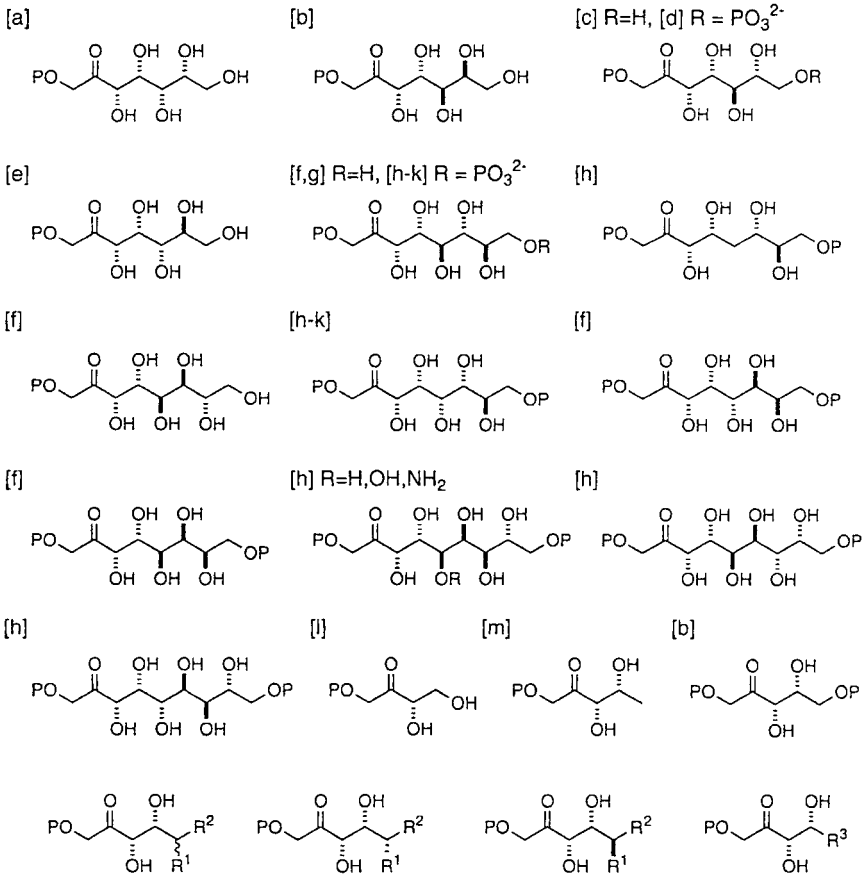


Figure 14.1-9. Use of the "inversion strategy" to synthesize L-xylose and 2-deoxy-D-*arabino*-hexose.

(Fig. 14.1-8)[83b]. Using this approach, different functionalized cyclitols may become easily accessible.

FDP aldolase is a useful catalyst for the direct synthesis of ketose monosaccharides and their analogs (*vide supra*). However, a number of the important naturally-occurring carbohydrates are aldoses. Various FDP aldolase products can be isomerized to a mixture of the ketose and aldose, and subsequently separated with Ca^{2+} or Ba^{2+} treated cation exchange resins. Another strategy involves the use of glucose isomerase (GI)[84], which catalyzes the isomerization of fructose (Fru) to glucose (Glc), and is used in the food industry for the production of high fructose corn syrup. GI also accepts analogs of Fru with modifications at positions 3, 5 and 6 as substrates[36]. Aldose analogs including 6-deoxy, 6-fluoro, 6-*O*-methyl and 6-azido-glucose have been synthesized using this FDP aldolase/GI methodology[36, 37]. However, not all FDP aldolase products are substrates for GI, and in the case of 5-deoxy-D-fructose, the equilibrium lies completely in the favor of the ketose. Furthermore, in the *inversion strategy* (Fig. 14.1-9)[42], monoprotected dialdehydes are used as substrates for FDP aldolase, generating protected aldehyde ketoses. The ketone group is then chemically or enzymatically stereoselectively reduced, and the aldehyde subsequently deprotected to produce the aldose. The strategy also places the vicinal diol produced in the aldol reaction in a position other than C3/C4. One enzyme suitable for the reduction is the NADH-dependant iditol dehydrogenase

Table 14.1-1. Products prepared from FDP aldolase-catalyzed reactions with DHAP.



R^1	R^2	Ref.	R^1	R^2	Ref.	R^1	R^2	Ref.	R^3
H	THPO	[n]	H	$CH_3CH(OH)$	[r]	BnO	CH_3	[n]	HCO [o]
H	BzO	[n]	H	$CF_3CONHCH_2$	[r]	Ph	CH_3	[n]	HOCO
H	CH_3CH_2	[n,o]	H	$(CH_3)_2C(OH)$	[r]	OH	N_3CH_2	[y,z]	$PhCH_2$
H	CH_3	[n,p]	H	CH_3OCH_2	[p]	OH	CH_3	[w]	$HCO(CH_2)_3$
H	$(EtO)_2PO$	[o]	H	$MeO_2CCH(NHAc)$	[s]	$CH_2{=}CHCH_2$	$HOCH_2$	[r]	BzO
H	Ph	[o]	OH	FCH_2	[q]	CH_3O	$HOCH_2$	[n]	
H	$OHC(CH_2)_3$	[o]	OH	CH_3	[t,u]	OH	$HOCH_2$	[r,w]	
H	$HOCH_2$	[q]	OH	$HOCH_2$	[q,v]	OH	N_3CH_2	[y,z]	
H	CbzNH	[r]	OH	CH_3CH_2	[w]	OH	CH_3	[v]	
H	$CbzNHCH_2$	[r]	OH	N_3CH_2	[x,y,z]				

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(IDH) from *Candida utilis*, also known as sorbitol or polyol dehydrogenase[35, 85]. Reduction of the ketone occurs to give the alcohol with (*S*)-stereochemistry. The corresponding (*R*)-alcohol was obtained by non-stereoselective reduction of the ketone with $NaBH(OAc)_3$' and the (*S*)-epimer was selectively removed by IDH-catalyzed oxidation. L-Xylose and 2-deoxy-D-*arabino*-hexose were synthesized by each of these two processes, respectively.

Other aldose/ketose isomerases with different substrate specificity have been cloned and overexpressed[86], including Fuc isomerase (Fuc I, EC 5.3.1.3) and Rha isomerase (Rha I, EC 5.3.1.14). Fuc isomerase, in combination with Fuc 1-P aldolase or Rha 1-P aldolase, has been used to prepare L-glucose, L-galactose, L-fucose, and derivatives from the corresponding L-glyceraldehyde derivatives and DHAP[87].

RAMA has been the most popular synthetic aldolase, due to its commercial availability. Notably, no significant differences in substrate specificity or stereoselectivity between FDP aldolases from different sources have been observed[88]. However, it is still important to verify this, especially for the type II aldolases which operate by a different mechanism. In fact, the type II aldolase from *E. coli*, which has been subcloned and overexpressed[27], has the potential to supplant RAMA as the FDP aldolase of choice for synthesis. It has enhanced stability compared with RAMA (*vide supra*), and is available from a microbial as opposed to an animal source. Table 14.1-1 illustrates products prepared from FDP aldolase-catalyzed reactions with DHAP.

14.1.1.2
Fuculose 1-Phosphate (Fuc 1-P) Aldolase (E.C. 4.1.2.17), Rhamnulose 1-Phosphate (Rha 1-P) Aldolase (E.C. 4.1.2.19) and Ragatose 1,6-Diphosphate (TDP) Aldolase

Fuc 1-P aldolase, Rha 1-P aldolase, and TDP aldolase also use DHAP as the donor substrate in aldol condensation. Fuc 1-P aldolase catalyzes the reversible condensa-

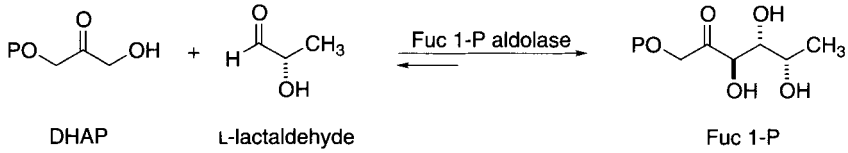


Figure 14.1-10. Aldol addition reaction catalyzed *in vivo* by Fuc 1-P aldolase.

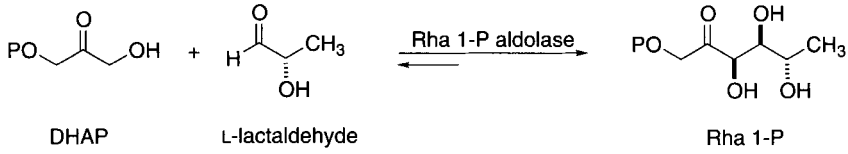


Figure 14.1-11. Aldol addition reaction catalyzed *in vivo* by Rha 1-P aldolase.

Figure 14.1-12. Aldol addition reaction catalyzed *in vivo* by TDP aldolase.

tion of DHAP and L-lactaldehyde to provide L-Fuc 1-P (Fig. 14.1-10). With the same substrates, Rha 1-P aldolase produces L-Rha 1-P (Fig. 14.1-11). Both of these enzymes are type II aldolases and are found in many microorganisms[89]. Both *E. coli* enzymes have been cloned, overexpressed in *E. coli*, and purified[90–92]. TDP aldolase, a type I aldolase involved in the galactose metabolism of *cocci*, catalyzes the reversible condensation of D-Gly 3-P with DHAP to give D-TDP (Fig. 14.1-12), and has also been cloned and overexpressed[92].

Both Fuc 1-P and Rha 1-P aldolase show specificity with regard to the aldehyde component, generating vicinal diol units of D-*erythro* and L-*threo* configurations, respectively (Fig. 14.1-13)[90–93]. While the stereospecificity for the absolute (3*R*)-configuration is mechanism-based, the configuration at C4 is somewhat substrate dependent. However, these two aldolases also show significant kinetic preference for the L-enantiomer of 2-hydroxyaldehydes (<95 : 5), facilitating resolution of racemic mixtures of these compounds (Fig. 14.1-14)[86]. Both enzymes have been used in the synthesis of rare ketose 1-phosphates[86], azasugars, and deoxyazasugars[54, 64, 94, 95]. Rha 1-P has also been employed in the synthesis of bicyclic carbohydrate structures[96].

Fuc 1-P and Rha 1-P aldolases have also been utilized in whole cell systems with DHA and catalytic inorganic aresenate[97]. With L-lactaldehyde as the substrate in the Rha 1-P aldolase reaction, the aldol product L-rhamnulose was subsequently isomerized to L-rhamnose, catalyzed by rhamnose isomerase. No such isomerization was observed with L-xylulose, the corresponding aldol product using glycolaldehyde as the substrate. Recent studies have since shown that both rhamnose and fucose isomerase require fixed stereochemistry only up to C3 for aldohexose substrates;

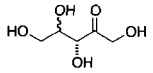
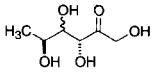
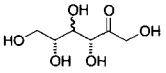
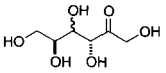
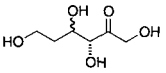
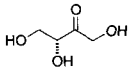
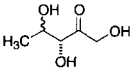
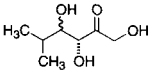
Aldehyde substrate	Product	Aldolase	Diastereomeric Ratio *trans* : *cis*	Relative Rate (%)
glycolaldehyde		Fuc 1-P	<3 : 97	38
		Rha 1-P	>97 : 3	43
L-lactaldehyde		Fuc 1-P	<3 : >97	100
		Rha 1-P	>97 : <3	100
D-Gly		Fuc 1-P	<3 : > 97	28
		Rha 1-P	>97 : <3	42
L-Gly		Fuc 1-P	<3 : >97	17
		Rha 1-P	>97 : <3	41
3-hydroxy-propionaldehyde		Fuc 1-P	<3 : >97	11
		Rha 1-P	>97 : <3	29
formaldehyde		Fuc 1-P	-	44
		Rha 1-P	-	22
acetaldehyde		Fuc 1-P	5 : 95	14
		Rha 1-P	69 : 31	32
i-butyraldehyde		Fuc 1-P	30 : 70	20
		Rha 1-P	97 : 3	22

Figure 14.1-13. Acceptor substrate specificity and diastereoselectivity of Fuc 1-P and Rha 1-P aldolase.

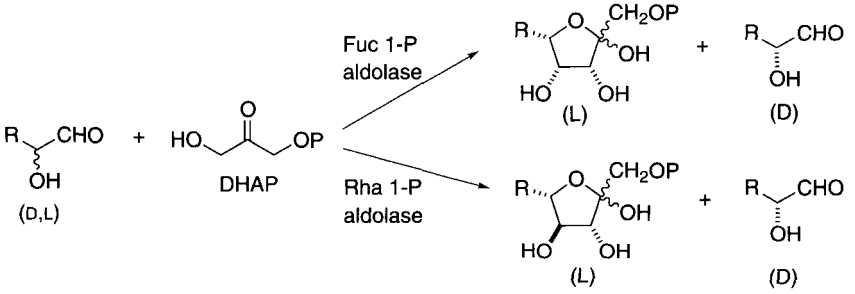


Figure 14.1-14. Kinetic resolution of 2-hydroxyaldehydes using Fuc 1-P and Rha 1-P aldolase.

(2*R*,3*R*) for rhamnose isomerase and (2*S*,3*R*) for fucose isomerase[86]. A sequential application of Fuc 1-P aldolase and fucose isomerase was employed for the preparation of L-fucose analogs[98].

TDP aldolase has been isolated from several sources[99]. The enzyme from *E. coli* has a narrow pH profile with an optimum at pH 7.5, but still displays acceptable

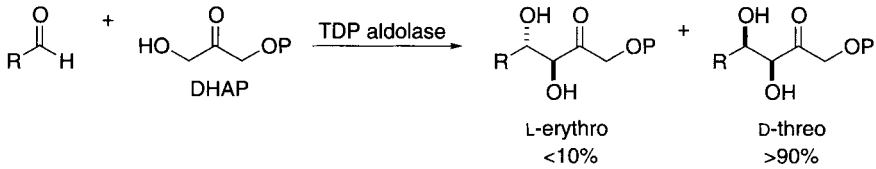


Figure 14.1-15. Diastereoselectivity of TDP aldolase.

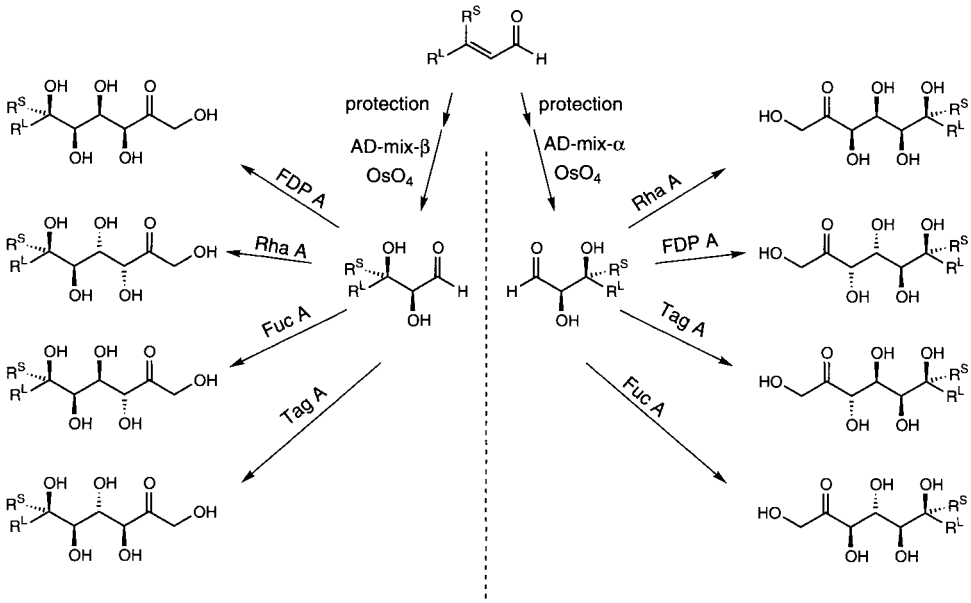


Figure 14.1-16. Product stereochemistries generated by the four complementary DHAP aldolases.

activity within pH 6.5–7. TDP aldolase accepts a variety of substrates, including glycoaldehyde, D- and L-glyceraldehyde, acetaldehyde, and *iso*butyraldehyde[100]. However, a diastereomeric mixture of products is generally formed. Also, only with the natural D-substrate does the major product (D-TDP) have the tagatose configuration (Fig. 14.1-15). Owing to this lack of stereoselectivity, TDP aldolase is not as synthetically useful as the other FDP aldolases. However, with suitable protein engineering, this may change in the future.

The four DHAP-utilizing aldolases generate all four stereochemical permutations of the vicinal diol at C3/C4 of the ketose product, and can be used to generate all four stereoisomers of a desired product (Fig. 14.1-16). In this manner, these enzymes have been utilized for the synthesis of sLe^x mimetic side chains[81], among other targets.

14.1.1.3
Synthesis of Dihydroxyacetone Phosphate (DHAP)

All four aldolases described previously use DHAP as the donor substrate, and several approaches have been taken towards its synthesis[101–105]. Enzymatic *in situ* generation of DHAP from FDP can be accomplished using FDP aldolase and triosephosphate isomerase (TI)[34]. FDP aldolase catalyzes the retro-aldol reaction of FDP to give D-Gly 3-P and DHAP, and TI catalyzes the conversion of D-Gly 3-P into DHAP. However, the overall reaction may not go to completion, depending on the thermodynamic stability of the product compared with FDP. The presence of FDP may also complicate product isolation. Another enzymatic method involves glycerol kinase-catalyzed phosphorylation of dihydroxyacetone (DHA) using ATP, with *in situ* regeneration of ATP[34, 101]. This procedure generates DHAP directly in high yield, but may be expensive for large-scale synthesis. Another method involves the generation of DHAP from phosphatidyl choline and DHA using phospholipases C and D[106]. DHAP and the corresponding phosphoramidate and phosphorothioate have been generated *in situ* enzymatically from the reduced form and used as substrates[104b].

DHA dimer can be phosphitylated chemically with $(PhCH_2O)_2PNEt_2$' and subsequently oxidized to the phosphate with H_2O_2 (Fig. 14.1-17)[102], or it can be phosphorylated directly with either $POCl_3$[103] or $(PhO)_2POCl$[104a]. Alternative efficient chemical syntheses of DHAP have also been reported[107]. These phosphate derivatives are all subsequently transformed into the stable dimer precursor of DHAP, which can easily be converted into DHAP by acid hydrolysis. The main drawback to preparing DHAP chemically is the lengthy synthetic procedure.

Alternatively, DHAP can be replaced by a mixture of DHA and inorganic arsenate[36, 97]. DHA reacts reversibly with inorganic arsenate ($k \sim 2.4 \times 10^{-3}$ $M^{-1}s^{-1}$) to form dihydroxyacetone arsenate, an analog of DHAP and a donor substrate for aldolases. In fact, in the presence of RAMA, triose phosphate isomerase, and inorganic arsenate, DHA was converted into D-Fru in almost quantitative yield[97]. However, reaction rates tend to be very slow, and low yields of aldol products are often obtained. Inorganic vanadate and DHA has also been investigated, but cannot be successfully utilized as a DHAP analog[97].

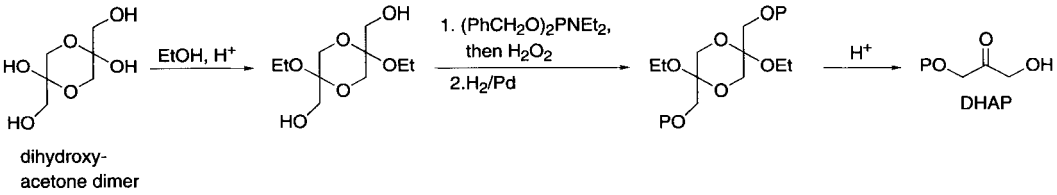


Figure 14.1-17. Chemical synthesis of DHAP.

14.1.2
Pyruvate/Phosphoenolpyruvate-Utilizing Aldolases

14.1.2.1
N-Acetylneuraminate (NeuAc) Aldolase (E.C. 4.1.3.3) and NeuAc Synthetase (E.C. 4.1.3.19)

NeuAc aldolase, or sialic acid aldolase, catalyzes the reversible condensation of pyruvate with *N*-acetylmannosamine (ManNAc) to form NeuAc (sialic acid; *N*-acetyl-5-amino-3,5-dideoxy-D-*glycero*-D-*galacto*-2-nonulopyranosonic acid) (Fig. 14.1-18)[108, 109].

The α-anomer of NeuAc serves as the aldolase substrate, even though the β-anomer predominates in solution. The initial products of aldol cleavage are α-ManNAc and pyruvate[110]. *In vivo*, the enzyme has a catabolic function, with an equilibrium constant for the retro-aldol reaction of 12.7 M^{-1}. However, for synthetic purposes, the production of the aldol product can be achieved using excess pyruvate[109]. NeuAc aldolase is a Schiff base-forming type I aldolase and has been isolated from both bacteria and animals. The optimum pH for activity is 7.5, although it retains activity between pH 6 and 9, and is stable under oxygen[109, 111]. The enzymes from *Clostridia* and *E. coli* are now commercially available (Toyobo), and that from *E. coli* has been cloned and overexpressed in *E. coli*[112]. It can be used free in solution, in immobilized form[113–116], or enclosed in a dialysis membrane[117].

Good conversion (~90%) of ManNAc to NeuAc has been achieved using the isolated enzyme, and purification can be achieved by decomposing excess pyruvate with pyruvate decarboxylase[118], or acid-catalyzed esterification of the products[119]. The need for excess pyruvate and purification of NeuAc can be circumvented by coupling the synthesis of NeuAc to a more thermodynamically stable process. For example, the NeuAc aldolase reaction can be coupled to a sialyltransferase reaction to produce sialyloligosaccharides[120]. Another variant of this process used a mixture of ManNAc and GlcNAc, whereby the GlcNAc was epimerized to ManNAc chemically[121, 122] or enzymatically[123, 124].

Extensive substrate specificity studies have indicated that only pyruvate is acceptable as the NeuAc aldolase donor substrate[116]. However, this enzyme has broad acceptor specificity, and over sixty aldoses have been characterized as substrates. Substitutions at C2, 4 and 6 of ManNAc are allowed, with only a slight preference for absolute stereochemistry at C4, 5 and 6[118–120, 125–130]. Some pentoses and their analogs are also substrates, although 2 and 3 carbon molecules are not accepted.

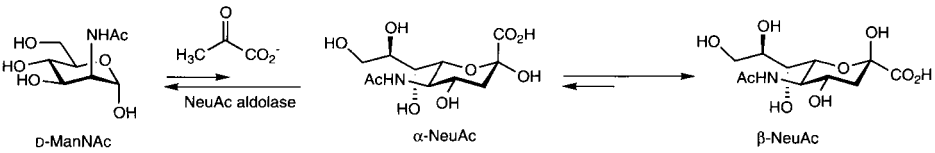


Figure 14.1-18. Aldol addition reaction catalyzed *in vivo* by NeuAc aldolase.

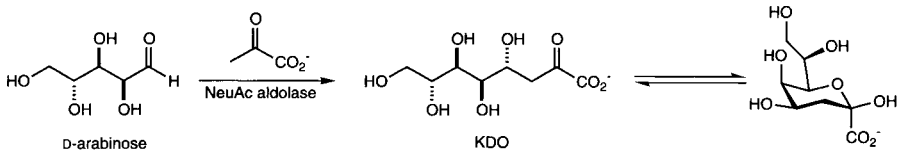


Figure 14.1-19. Synthesis of KDO using NeuAc aldolase.

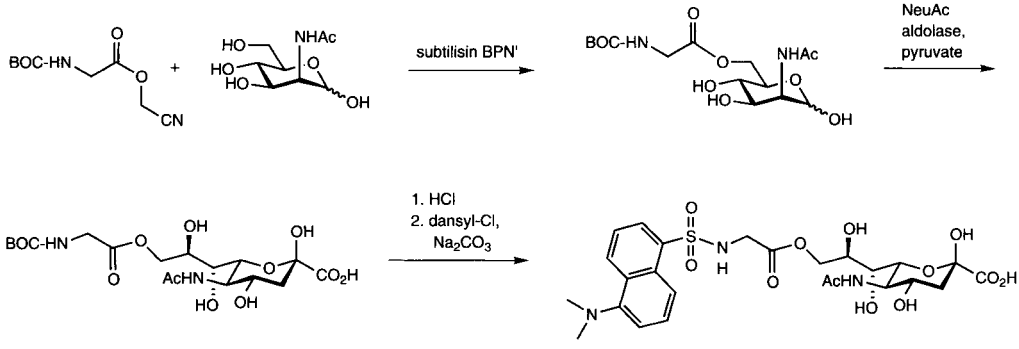


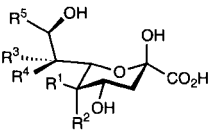
Figure 14.1-20. Synthesis of 9-*O*-acetylNeuAc by combined use of subtilisin and NeuAc aldolase.

The stereoselectivity of NeuAc aldolase is unusual, as it is thermodynamically controlled. With the natural substrate D-ManNAc, attack occurs exclusively on the *si* face of the carbonyl group. However, a mixture of KDO and 4-*epi*-KDO was obtained when D-arabinose was used as a substrate[128], and a complete reversal of diastereoselectivity was observed with L-mannose and 6-deoxy-L-mannose[118, 119, 131]. With these latter aldehydes, exclusive attack on the *re* face of each carbonyl group gives the more thermodynamically favored products. NeuAc aldolase has been investigated as a catalyst for the condensation of various pentoses and hexoses, and enzyme stereoselectivity characterized[132]. Furthermore, KDN has been produced on a 100 gram scale from pyruvate and D-mannose in a crystallized yield of 75%[133].

NeuAc, derivatives thereof, and polysialic acids play important roles in cell-cell adhesion and communication in bacterial and mammalian systems[134, 135]. The wide substrate specificity and ready availability of NeuAc aldolase provide the opportunity for the synthesis of many sialic acid derivatives. Azasugar analogs of NeuAc were synthesized using the 3-deoxy-3-azido analogs of ManNAc, mannose, and glucosamine as acceptor substrates[136]. Similarly, KDO was produced using D-arabinose as the acceptor (Fig. 14.1-19).

A facile synthesis of 9-*O*-acetyl-NeuAc[114] has been accomplished via regioselective irreversible acetylation of ManNAc catalyzed by subtilisin, followed by NeuAc aldolase-catalyzed condensation of the resulting 6-*O*-acetyl-ManNAc with pyruvate[116]. A 9-*O*-glycyl-NeuAc derivative was prepared in a similar fashion, and was further converted into a fluorescent derivative (Fig. 14.1-20)[137].

A large number of NeuAc derivatives modified at the 5-amino group[138], the C7-position[139], or the C9-position[138e, 140] have been reported. Many other compounds



R^1	R^2	R^3	R^4	R^5	Ref.
AcNH	H	OH	H	CH_2OH, CH_2OAc, CH_2N_3, CH_2F, CH_2OMe, $CH_2OCOCHOHCH_3$	1,2,3, 5,11
OH	H	OH	H	CH_2OH, CH_2OAc, H	7,4,10
OH	H	H	H, F	CH_2OH, CH_2F	8,9
H	H,OH	OH	H	CH_2OH	10,8
Ph	H	OH	H	CH_2OH	10
N_3	H	OH	H	CH_2OH	8

Figure 14.1-21. NeuAc analogs synthesized using NeuAc aldolase.

have been prepared using this aldolase (Fig. 14.1-21)[47, 141–144], including an α-methyl ketoside of 5-amino-NeuAc[141], and polyacrylamides bearing pendant α-sialoside groups[143] or *C*-linked sialosides[144]. The latter strongly inhibit agglutination of erythrocytes by the influenza virus.

The synthesis of NeuAc *in vivo* is accomplished using NeuAc synthetase[111]. This aldolase catalyzes the irreversible condensation of PEP and *N*-acetylmannosamine. Although this enzyme has not yet been isolated and characterized, it may prove synthetically useful in the future.

14.1.2.2
3-Deoxy-D-*manno*-2-octulosonate Aldolase (E.C. 4.1.2.23) and 3-Deoxy-D-*manno*-2-octulosonate 8-Phosphate Synthetase (E.C. 4.1.2.16)

3-Deoxy-D-*manno*-2-octulosonate aldolase, also known as 2-keto-3-deoxyoctanoate (KDO) aldolase, catalyzes the reversible condensation of pyruvate with D-arabinose to form KDO (Fig. 14.1-22). KDO and its activated form CMP-KDO are key intermediates in the synthesis of the outer membrane lipopolysaccharide (LPS) of Gram-negative bacteria[145]. Inhibitors of LPS biosynthesis or LPS binding protein[146] therefore serve as antimicrobial agents[147–151]. KDO aldolase has a catabolic function, with an equilibrium constant for degradation of 0.077 M. It has been isolated and purified from *E. coli*[152] and *Aerobacter cloacae*[153]. Preliminary investigations on this enzyme showed high specificity for KDO in the direction of cleavage, whereas the condensation reaction proceeded with some flexibility. Several unnatural substrates, including D-ribose, D-xylose, D-lyxose, L-arabinose, D-arabinose 5-phosphate and *N*-acetylmannosamine were reported to be weak substrates (relative rate >5% cf. D-arabinose[152]). Studies on the substrate specificity of KDO aldolase from *Aureobacterium barkerei* strain KDO-37–2, have indicated that this enzyme

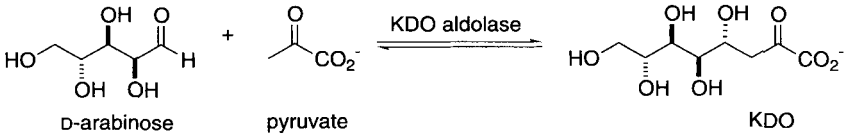


Figure 14.1-22. Aldol addition reaction catalyzed *in vivo* by KDO aldolase.

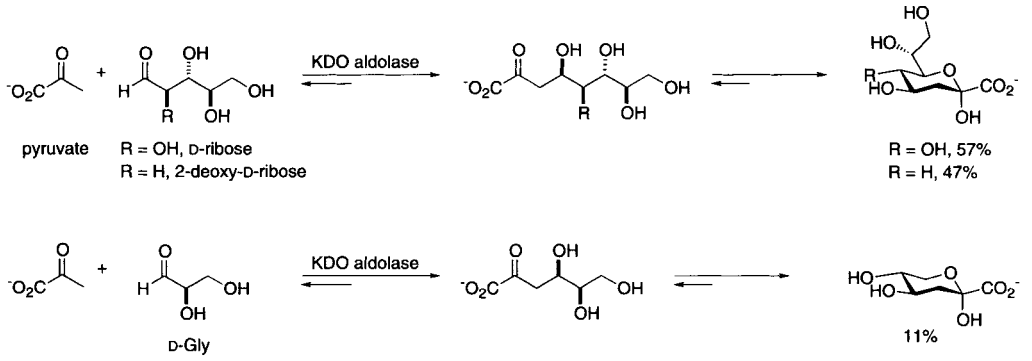


Figure 14.1-23. KDO aldolase-catalyzed synthesis of carbohydrates.

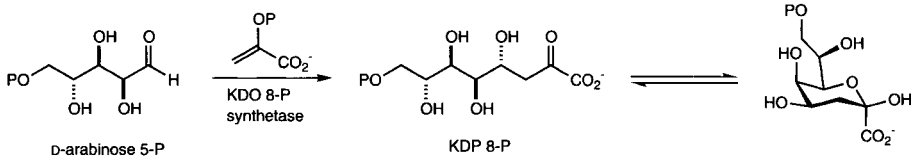


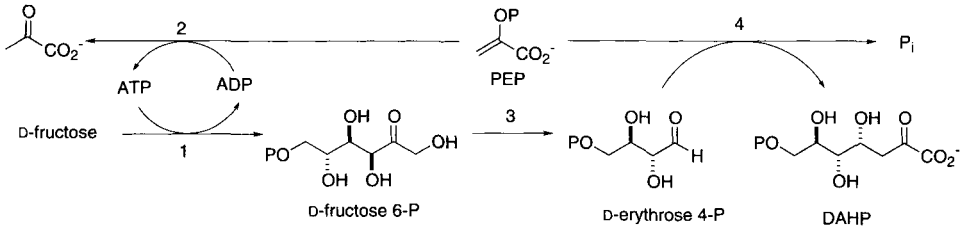
Figure 14.1-24. Aldol addition reaction catalyzed *in vivo* by KDO 8-P synthetase.

widely accepts trioses, tetroses, pentoses and hexoses as substrates[154]. The best substrates have (*R*)-configuration at C3, with the substituent at C2 having little effect. Several aldol addition reactions have been conducted on a preparative scale, including the synthesis of KDO itself, which was obtained in 67% yield (Fig. 14.1-23). In each case, attack of the pyruvate took place on the *re* face of the carbonyl group of the acceptor substrate. Excess pyruvate can be decomposed with pyruvate decarboxylase to simplify the isolation[154].

3-Deoxy-D-*manno*-2-octulosonate 8-phosphate synthetase, also known as phospho-2-keto-3-deoxyoctanoate (KDO 8-P) synthetase, catalyzes the irreversible aldol reaction of PEP and D-arabinose 5-phosphate to give KDO 8-P (Fig. 14.1-24)[155]. The enzyme has been isolated from *E. coli* B[156] and *Pseudomonas aeruginosa*[157], and the *E. coli* enzyme has been cloned and overexpressed in *E. coli* and *Salmonella typhimurium*[158]. It has been used in the synthesis of KDO 8-P, using D-arabinose 5-phosphate generated either by hexokinase-catalyzed phosphorylation of arabinose[152], or an isomerase-catalyzed reaction of D-ribose 5-phosphate[157]. Studies indicate KDO-8-P is very specific for its natural substrates, although some KDO analogs may be accessible.

14.1.2.3
3-Deoxy-D-arabino-2-heptulosonic Acid 7-Phosphate (DAHP) Synthetase (E.C. 4.1.2.15)

In vivo, DAHP synthetase, also known as phospho-2-keto-3-deoxyheptanoate synthetase, catalyzes the synthesis of DAHP from PEP and D-erythrose 4-phosphate[159]. DAHP is a key intermediate in the shikimate pathway for the biosynthesis of



1. Hexokinase, 2. Pyruvate kinase, 3. Transketolase + D-ribose 5-P, 4. DAHP synthetase

Figure 14.1-25. Multi-enzyme synthesis of DAHP.

aromatic amino acids in plants[160]. The enzyme has been cloned[161] and used to synthesize DAHP (Fig. 14.1-25)[162]. In this synthesis, D-erythrose 4-phosphate was generated *in situ* from Fru 6-P, catalyzed by transketolase in the presence of D-ribose 5-phosphate. Fru 6-P was generated from D-Fru and ATP, catalyzed by hexokinase in the presence of an ATP regeneration system. In general, it is more efficient and economical to use whole cells containing a DAHP synthetase plasmid[163]. Such a system also provides the necessary enzymes for the synthesis of DHAP. Recently, DAHP synthase purified and overexpressed in *E. coli* has been characterized with respect to substrate specificity, and catalyzes the condensation of PEP with ribose-5-phosphate, deoxyribose-5-phoshate, and arabinose-5-phosphate[164]. This enzyme has also been employed as a component of a biocatalytic process for large-scale production of vanillin from glucose[165].

14.1.2.4
2-Keto-4-hydroxyglutarate (KHG) Aldolase (E.C. 4.1.2.31)

In vivo, KHG aldolase catalyzes the reversible condensation of pyruvate and glyoxylate to form KHG (Fig. 14.1-26)[166, 167]. This enzyme participates in the terminal step of mammalian catabolism of L-hydroxyproline[166]. The enzymes isolated and purified from bovine liver and *E. coli* are both type I aldolases. Limited substrate

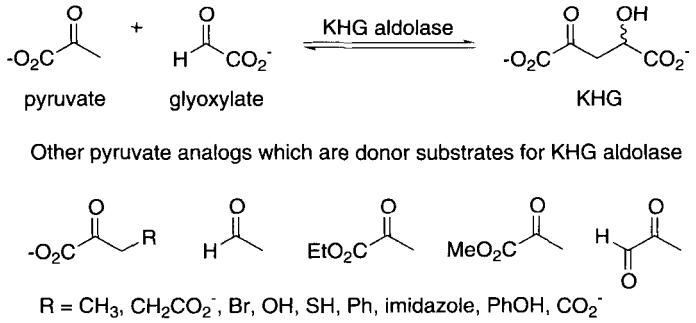
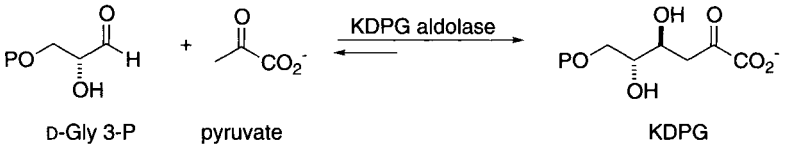


Figure 14.1-26. Aldol addition reaction catalyzed *in vivo* by KHG aldolase and the donor substrate specificity of this enzyme.

specificity studies on KHG aldolase from bovine liver indicate that it accepts both enantiomers of KHG equally well, and also cleaves 2-keto-3-deoxyglucarate, 2-keto-4,5-dihydroxyvalerate, and oxaloacetate[167]. In the condensation direction, this enzyme is relatively specific for glyoxylate, although it does accept other pyruvate derivatives[168]. The enzyme from *E. coli* prefers the natural substrate [KHG with (*S*)-configuration] and also cleaves 2-keto-4-hydroxybutyrate and oxaloacetate[169]. Using the *E. coli* enzyme, both L- and D-4-hydroxy-2-ketoglutarate have been prepared on a millimole scale[170]. In the condensation reaction, glyoxylate can be replaced with glyoxaldehyde, formaldehyde, acetaldehyde, and formic acid, while pyruvate can be substituted by α-ketobutyrate and bromopyruvate.

14.1.2.5
2-Keto-3-deoxy-6-phosphogluconate (KDPG) Aldolase (E.C. 4.1.2.14)

In vivo, KDPG aldolase catalyzes the reversible condensation of pyruvate with D-Gly 3-P to form KDPG (Fig. 14.1-27). The equilibrium constant lies in favor of the aldol addition ($K \sim 10^3$ M^{-1}). KDPG aldolase accepts a number of unnatural acceptor aldehydes, although at rates much lower than the natural substrate[171]. Various sources of KDPG aldolase have been investigated as C – C bond forming catalysts in organic synthesis[172], such as for the synthesis of non-carbohydrate components of the nikkomycin natural products[173]. The related enzyme KDPGal aldolase has also been utilized for similar purposes sp[174]. Unlike other aldolases, simple aliphatic aldehydes are not KDPG aldolase substrates. However, other than the presence of polar functionality at C2 or C3, there appears to be no other structural requirement for the acceptor aldehyde. These studies also demonstrate that KDPG aldolase stereospecifically generates the new stereocenter at C4 with (*S*)-configuration. Furthermore, by using the technique of directed evolution, KDPG aldolase has been altered with respect to its acceptor enantioselectivity and phosphate requirement to accept non-phosphorylated enantiomeric aldehydes[175].



Other acceptor substrates of KDPG aldolase

Acceptor	V_{rel}	Acceptor	V_{rel}
nitropropanal	200	erythrose	1.5
chloroacetaldehyde	120	glycoaldehyde	1.5
D-glyceraldehyde	100	benzaldehyde	0
D-lactaldehyde	27	butyraldehyde	0
ribose 5-P	5	ribose	0

Figure 14.1-27. Aldol addition reaction catalyzed *in vivo* by KDPG aldolase and the acceptor substrate specificity of this enzyme.

14.1.2.6
2-Keto-3-deoxy-D-glucarate (KDG) Aldolase (E.C. 4.1.2.20)

In vivo, KDG aldolase catalyzes the reversible reaction of pyruvate and tartronic acid semialdehyde to form KDG (Fig. 14.1-28). This aldolase has been found in various bacteria and the enzyme from *E. coli* has been isolated and purified[176]. KDG aldolase accepts several other aldehyde acceptor substrates, including glycoaldehyde, glyoxylate, and D- and L-glyceraldehyde. It has been used to synthesize 2-keto-3-deoxy-D-gluconate on a preparative scale[177].

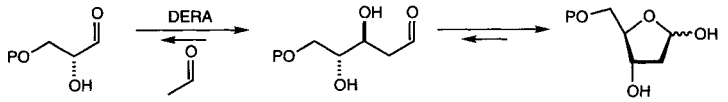
14.1.3
2-Deoxyribose 5-phosphate Aldolase (DERA) (E.C. 4.1.2.4)

DERA[178] is unique among the aldolases, in that the donor of the aldol reaction is an aldehyde, rather than a ketone. *In vivo,* the enzyme catalyzes the reversible condensation of acetaldehyde and D-Gly 3-P to form D-2-deoxyribose 5-phosphate, with an equilibrium constant in the cleavage direction of 2×10^{-4} M (Fig. 14.1-29). It is a type I aldolase, and has been isolated from animal tissues[179] and microorganisms[180]. The *E. coli* gene encoding DERA has been sequenced[181], subcloned, and the enzyme overexpressed in *E. coli*[182–184]. At 25 °C and pH 7.5, DERA is fairly stable (70% activity retained after 10 days).

A number of unnatural substrates are accepted by DERA (Fig. 14.1-29), and it generates (*R*)-configured chiral centers. DERA from *L. plantarum*[185] accepts various acceptor substrates including L-Gly 3-P, D-erythrose 4-phosphate, glycoaldehyde phosphate, D-ribose 5-phosphate, D,L-glyceraldehyde, D-erythrose, and D-threose[186]. Only propionaldehyde can weakly replace acetaldehyde as the donor. The *E. coli* enzyme[182] accepts acetaldehyde, propionaldehyde, acetone, fluoroacetone, aliphatic aldehydes, sugars, and sugar phosphates as acceptor substrates. However, the rates of the aldol reactions are very slow (0.4–1% *cf.* the natural substrates). More recently, DERA has been used to obtain key intermediates in the synthesis of the epothilone class of natural products[188]. Several syntheses of azasugars conducted using DERA are illustrated in Fig. 14.1-30.

When acetaldehyde is used as the donor, the products from the DERA-catalyzed reaction are aldehydes, capable of being acceptor substrates for a second aldol condensation (Fig. 14.1-31)[187]. For example, when α-substituted acetaldehydes were employed as substrates, products of the first aldol condensation could not cyclize to a hemiacetal, and the products reacted with a second molecule of acetaldehyde to form 2,4-dideoxyhexoses. These products could then cyclize to stable

Figure 14.1-28. Aldol addition reaction catalyzed *in vivo* by KDG aldolase.



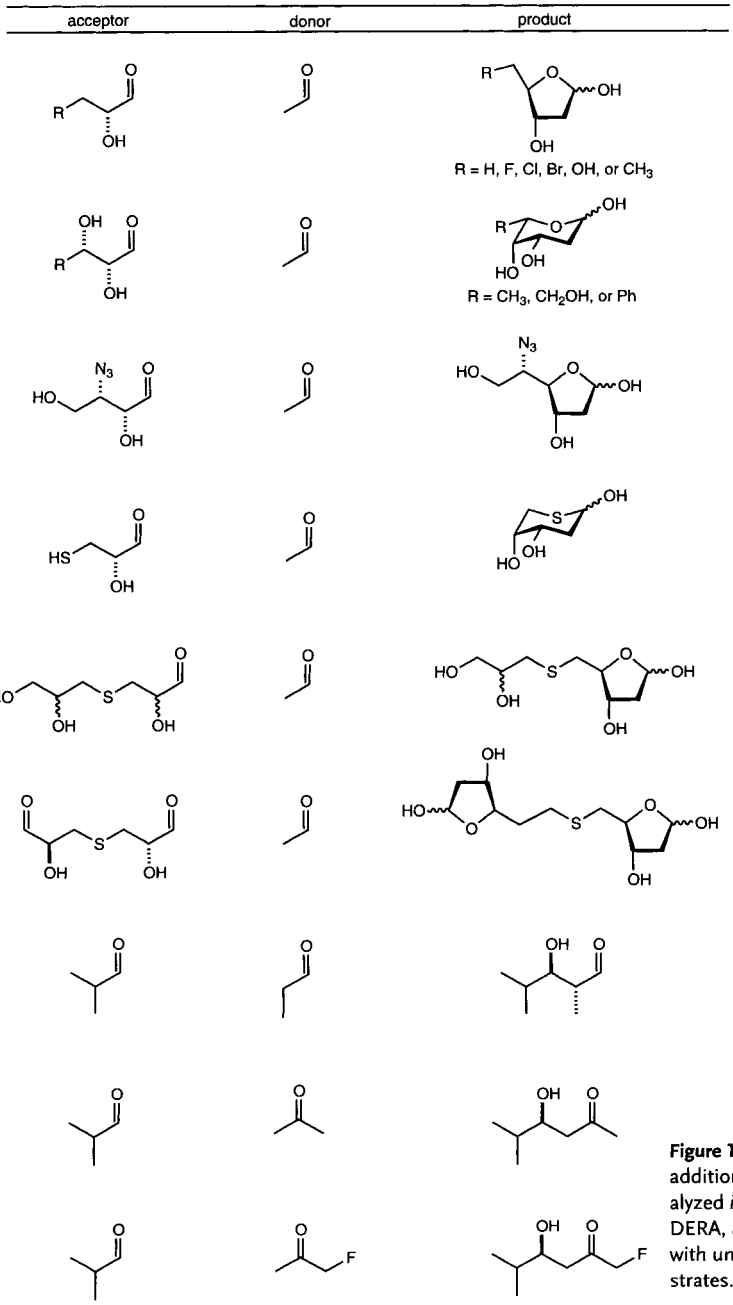
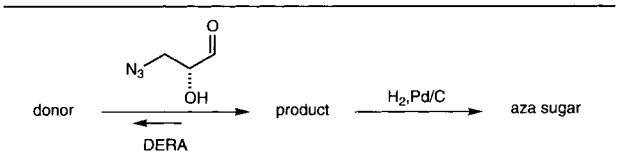


Figure 14.1-29. Aldol addition reaction catalyzed *in vivo* by DERA, and reactions with unnatural substrates.



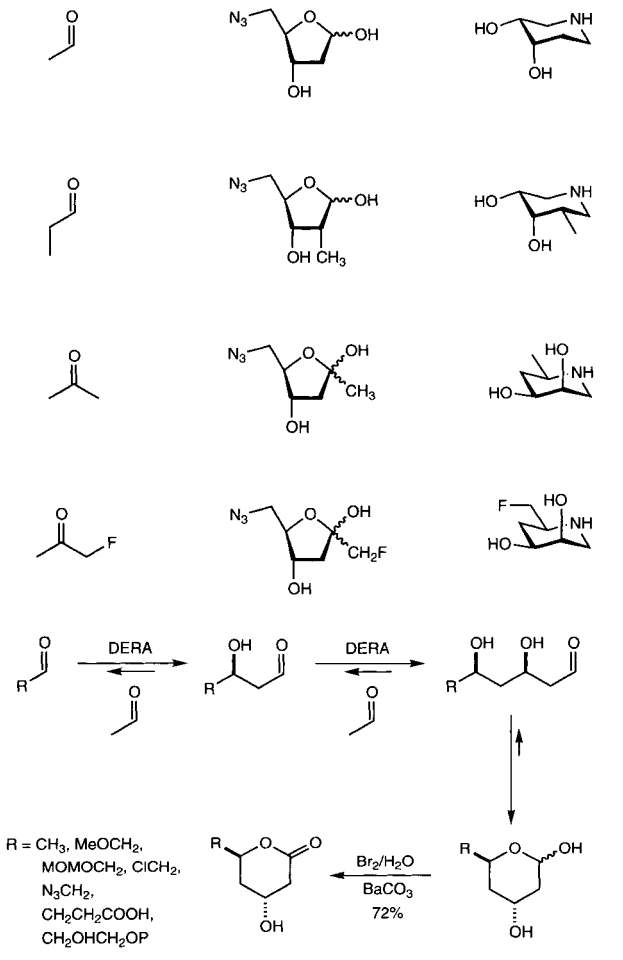


Figure 14.1-30. Syntheses of azasugars using DERA.

Figure 14.1-31. Sequential aldol reactions catalyzed by DERA.

hemiacetals, thus stopping the polymerization after two sequential aldol reactions. Conversion to chiral lactone derivatives of mevinic acids, which are active as cholesterol-lowering agents, could then be accomplished. The best substrate for the DERA-catalyzed sequential reaction appeared to be succinic semialdehyde (R = CH_2CH_2COOH) in which the carboxylic acid mimics the Gly 3-P phosphate group[184].

One-pot sequential aldol reactions were performed by combining DERA with FDP

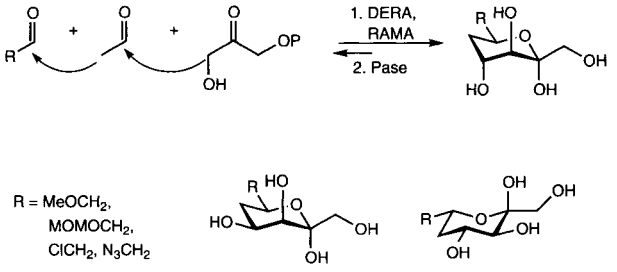


Figure 14.1-32. One-pot aldol reaction employing RAMA and DERA.

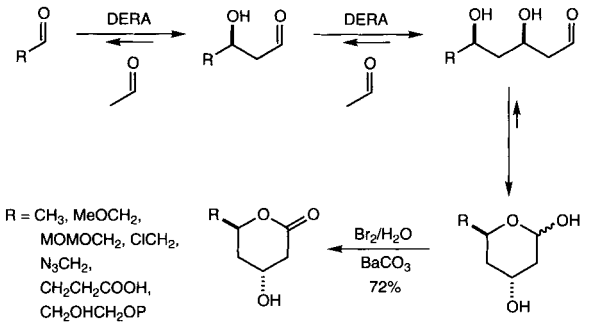


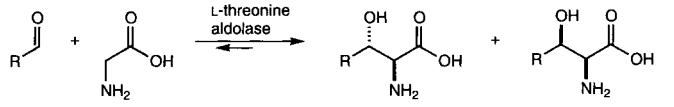
Figure 14.1-33. Tandem use of DERA and NeuAc aldolase.

aldolase (Fig. 14.1-32)[189, 190]. The products of these reactions are 5-deoxy ketoses with three substituents in axial positions. Owing to the formation of these thermodynamically unfavored products at long reaction times, some inversion of the usual stereochemistry of both DERA and FDP aldolase was observed. Combination of DERA and NeuAc-aldolase catalysis gave sialic acid derivatives (Fig. 14.1-33)[189]. In this case, however, one-pot synthesis was not possible, due to the incompatibility of the reaction conditions for the two aldolases.

Glycine-dependent Aldolases

The glycine-dependent aldolases, including serine hydroxymethyltransferases (SHMT) and threonine aldolases, are pyridoxal 5-phosphate-dependent enzymes which catalyze the reversible aldol reaction of glycine with an aldehyde acceptor to form a β-hydroxy-α-amino acid[191]. *In vivo* SHMT (EC 2.1.2.1) catalyzes the condensation of glycine and formaldehyde to give L-serine, and requires the cofactor tetrahydrofolate[191a]. SHMT has been used for the resolution of racemic *erythro* β-hydroxy α-amino acids, the large-scale synthesis of L-serine[192, 193], and the production of 2-amino-3-hydroxy-1,6-hexanedicarboxylic acid[194]. Although SHMT is selective for the L-configuration at the α-center, it generally displays poor *erythro-threo* discrimination, resulting in product mixtures[195, 196].

Threonine aldolases catalyze the reversible aldol reaction between glycine and acetaldehyde to give threonine (Fig. 14.1-34), and both D- and L-Thr aldolases have been reported. The substrates for the L-threonine aldolases (E.C. 4.1.2.5) are also substrates for L-SHMT (*vide supra*). Many threonine aldolases also accept allo-



R	Yield (%)	Ratio (erythro:threo)
CH_3 —	38	93 : 7
Ph —	87	60 : 40
N_3CH_2 —	45-75	70 : 30 to 100 : 0
$BnOCH_2$ —	78	92 : 8
BnO CH_2 —	53	53 : 47
BnO O CH_2 —	45	92 : 8
PhS CH_2 —	80	50 : 50
O $O-CH_2$ — N O	10	86 : 14

Figure 14.1-34. Reaction catalyzed *in vivo* by L-Thr aldolase, and unnatural substrates.

threonine derivatives as substrates, sometimes preferably over compounds with the *threo* configuration[197, 198].

Threonine aldolases have been used extensively for the resolution of racemic β-hydroxy α-amino acids. For example, with a L-threonine aldolase isolated from *Streptomyces amakusaensis*, several racemic mixtures of 3-(*p*-substituted-phenyl-)serines were resolved to give the enantiomers with the D-*threo* stereochemistry in >95 % ee[199, 200]. Recently, both D-[201] and L-Thr aldolases[201, 202] have been used in the preparation of novel β-hydroxy-α-amino acids. In addition, D-threonine aldolase has been utilized to prepare a small molecule that acts as a gelator of organic solvents[203]. L-Threonine aldolase has been employed in the synthesis of fragments of the mycestericin class of natural products[204], as well as peptidic RNA mimetics[205]. L-Threonine aldolase (E.C. 4.1.2.5) from *Candida humicola* has been crystallized[206], and has been investigated for use in condensation reactions[198]. The enzyme accepted a broad range of aldehydes, but in general mixtures of L-*erythro* and L-*threo* products were obtained, with the L-*erythro* configuration being the preferred one (Fig. 14.1-34).

When hydroxyaldehydes are employed as L-Thr aldolase substrates, complex product mixtures result. Protection of the hydroxyl groups prevents this, and allowes the preparation of C4-protected L-threonine and L-allothreonine derivatives. Acceptor aldehydes with an oxygen functionality at the α-position gave high *erythro/threo*

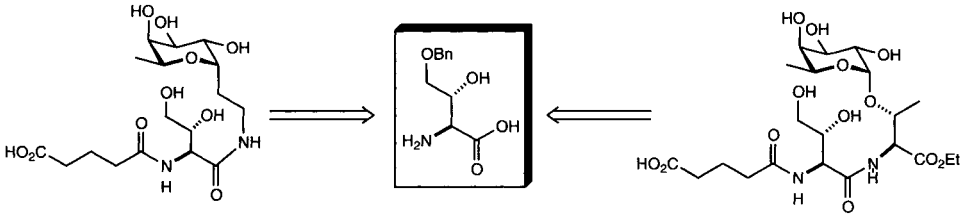


Figure 14.1-35. Use of L-Thr aldolase in the preparation of sLe^x mimetics.

ratios, a ratio which was reduced when the oxygen was in the β-position. Although α,β-unsaturated aldehydes did not serve as substrates, several thiophenol derived aldehydes were accepted, providing a route toward unsaturated amino acids. One L-Thr aldolase product, the 4-hydroxy-L-allothreonine derivative, has been used as a key intermediate in the synthesis of potent sialyl Le^x mimetics (Fig. 14.1-35) [207].

Other known aldolases whose substrate specificity remains to be examined are summarized in Table 14.1-2.

Catalytic Antibodies

In recent years, catalytic antibody technology has provided methods for developing new protein catalysts [208]. Monoclonal antibodies (mAbs) elicited against "transition-state" haptens catalyze reactions with remarkable rate accelerations. By appropriate antigen design, functional groups that perform general acid/base catalysis, nucleophilic/electrophilic catalysis, and catalysis by strain or proximity effects can be induced into the binding site of an antibody. Even reactions which are unfavorable or otherwise unattainable have been achieved using the catalytic antibody approach. Aldolase catalytic antibodies developed recently have the ability to match the efficiency of the natural aldolases while accepting a more diverse range of substrates.

Initial catalytic antibodies were developed to bind a primary amine cofactor as a mimic of the type I aldolases. The hapten designed mimicked the transition state the iminium ion, resulting in the production of an antibody that catalyzed the aldol condensation of acetone and aldehyde acceptors (Fig. 14.1-36) [209]. Even though no stereochemical information was built into the transition-state mimic, the antibody catalyzed stereoselective addition to the *si* face of the aldehyde.

The subsequent development phase, namely reactive immunization [210], involved

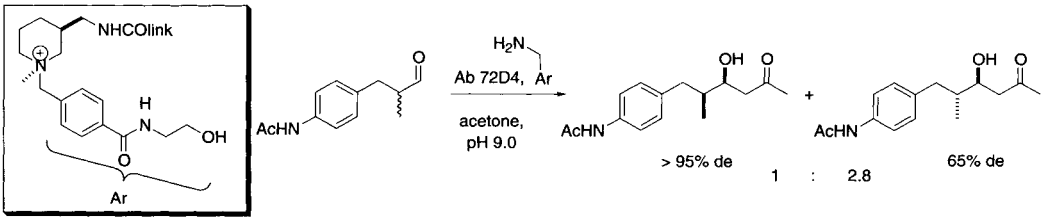
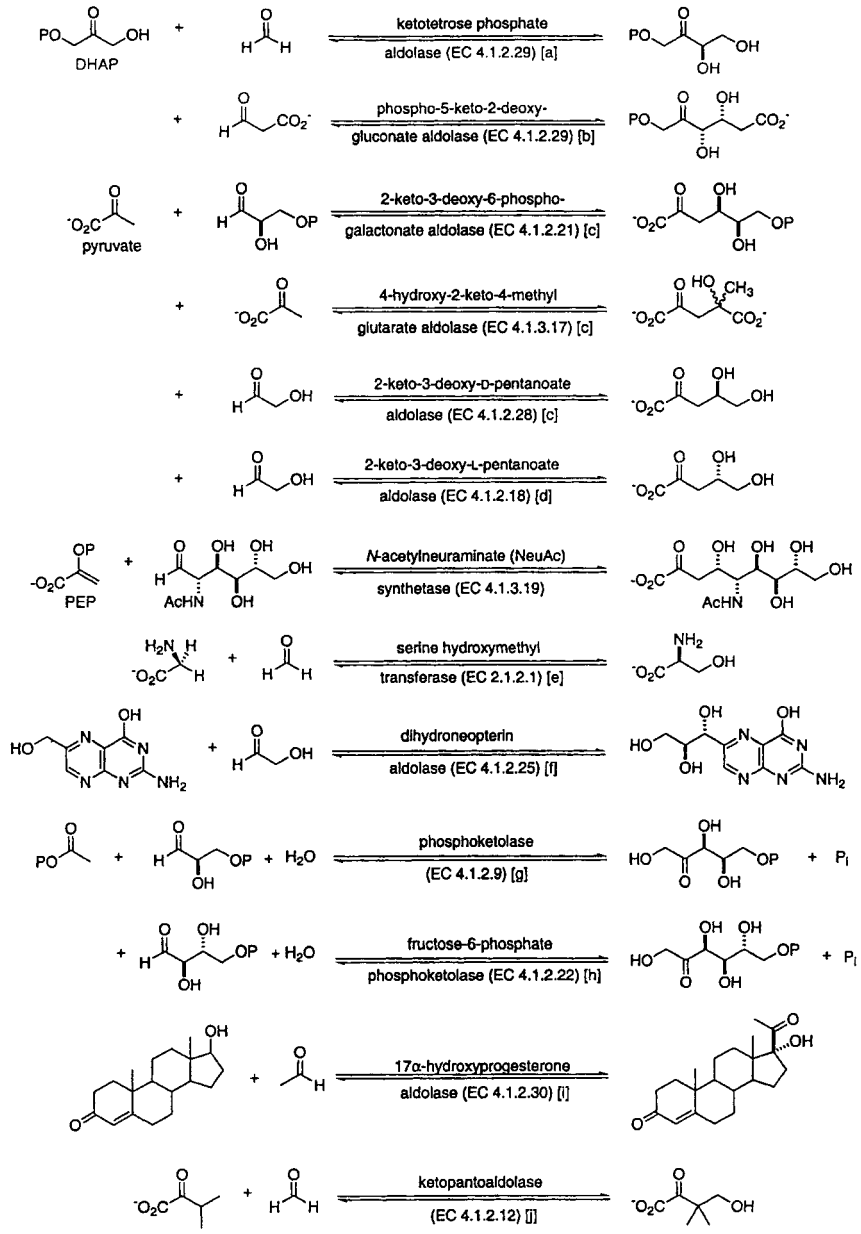


Figure 14.1-36. Aldol reaction catalyzed by catalytic antibody 72D4, and a transition-state hapten.

Table 14.1-2. Other aldolases and the reactions they catalyze *in vivo*.



a Isolated from rat liver, see: F. C. Charalampous, *Methods Enzymol.* 1962, 5, 283. Acetaldehyde, glycoaldehyde or glyceraldehyde cannot replace formaldehyde.

b W. A. Andeson, B. Magasanik, *J. Biol. Chem.* 1971, *246*, 5662.

c W. A. Wood in: *The Enzymes* (Ed.: P. D. Boyer), Academic Press, New York, **1970**; *Vol. VII*, p. 281.

d This enzyme also catalyzes the aldol addition of pyruvate with formaldehyde to give 4-hydroxy-2-oxobutyrate, originally thought to be catalyzed by hydroxyoxobutyrate aldolase (E.C. 4.2.1.1). Phenylpyruvate is also a donor substrate, while acetaldehyde, benzaldehyde and crotonaldehyde are not acceptor substrates, see: H. Hift, H. R. Mahler, *J. Biol. Chem.* 1952, *198*, 901.
e L. Schirch, *Adv. Enzymol.* 1982, *53*, 83. A multicopy plasmid containing the *E. coli* serine hydroxymethyl transferase was introduced to *Klebsiella aerogenes* for overexpression of the enzyme. The enzyme requires tetrahydrofolate (THF) and pyridoxal phosphate. THF first reacts nonenzymatically with formaldehyde to form *N*5,*N*10-methylene THF which is then accepted by the enzyme to form serine, see: B. K. Hamilton, H. Y. Hsiao, W. E. Swanm, D. M. Anderson, J. Delente, *J. Trends Biotechnology*, 1985, *3*, 64. This enzyme also catalyzes the reversible aldol reaction of glycine with acetaldehyde to give L-allothreonine, originally thought to be catalyzed by L-allothreonine aldolase (E.C. 4.1.2.6).
f J. B. Mathis, G. M. Brown, *J. Biol. Chem.* 1970, *245*, 3015. The reaction requires thiamine pyrophosphate and favors cleavage.
g E. C. Heath, J. Hurwitz, B. L. Horecker, A. Ginsberg, *J. Biol. Chem.* 1958, *231*, 1009. The reaction favors the cleavage of D-xylulose-5-phosphate. The enzyme from *Leuconostoc mesenteroides* also accepts fructose-6-phosphate, hydroxypyruvate and glycoaldehyde as substrates.
h E. Racker, *Methods Enzymol.* 1992, *5*, 276. The reaction favors degradation.
i D. E. Johnston, Y.-B. Chiao, J. S. Gavaler, D. H. Van Thiel, *Biochem. Pharm.* 1981, *30*, 1827.
j W. K. Maas, H. J. Vogel, *J. Bacteriol.* 1953, *65*, 388; E. N. McIntosh, M. Purko, W. A. Wood, *J. Biol. Chem.* 1957, *228*, 499.

raising antibodies against a β-diketone "chemical trap" to imprint the lysine-dependent type I aldolase mechanism in the active site (Fig. 14.1-37)[211]. The ε-amino group of a lysine side chain reacts with the β-diketone to give a β-ketoimine, which tautomerizes to the stable vinylogous amide. By using this method, two catalytic antibodies with aldolase selectivity, 38C2 and 33F12, were identified and subsequently shown to have remarkable scope[212]. The structure of 33F12 has been determined and shown to have the Schiff base forming Lys residue buried in a hydrophobic pocket at the base of the binding site[211].

Unlike natural aldolases, catalytic antibodies accept a wide range of ketone donor substrates (Fig. 14.1-38A). Small aliphatic ketones are well tolerated, but mixtures of products result with unsymmetrical ketones, due to reaction at both α-positions. α-Heteroatom-substituted ketones show much higher levels of regioselectivity, with reaction occurring almost exclusively at the carbon atom bearing the heteroatom. Interestingly, the regiochemistry of the reaction of fluoroacetone is opposite to that observed with the natural aldolase DERA, thus providing a complementary approach.

A wide variety of aldehydes serve as acceptors (Fig. 14.1-38B), including those that

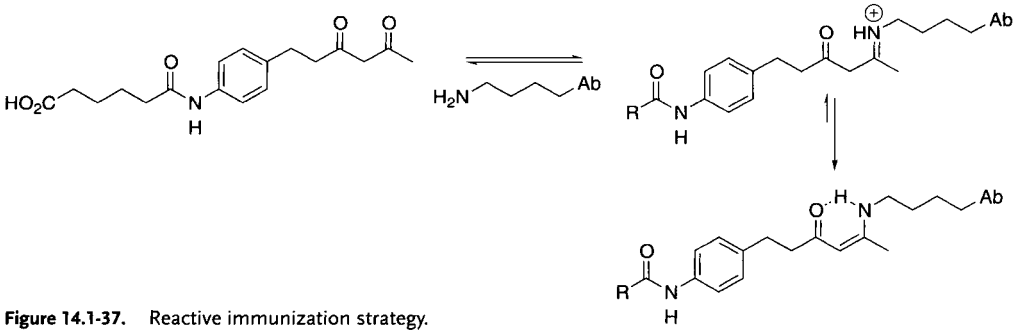


Figure 14.1-37. Reactive immunization strategy.

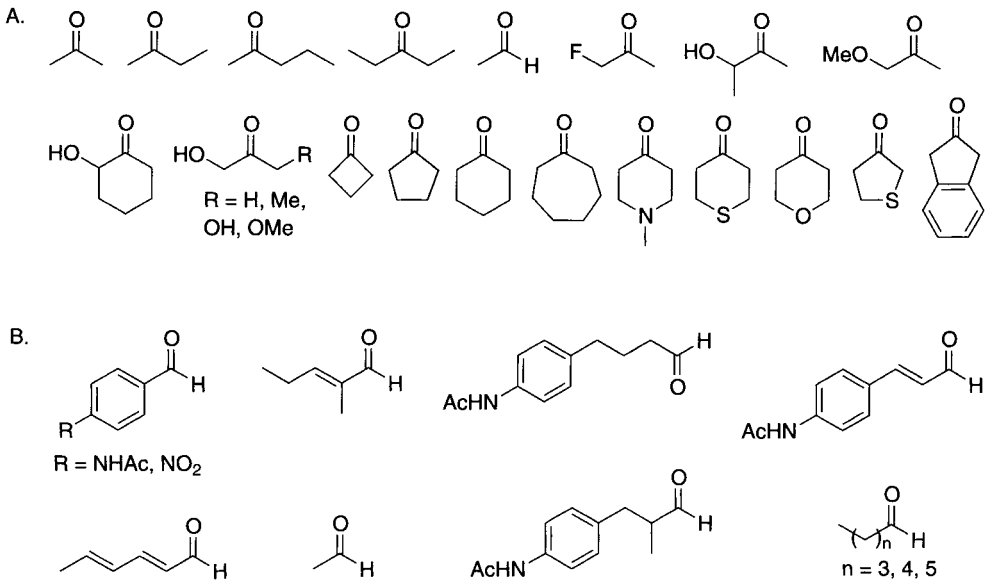


Figure 14.1-38. A, Catalytic antibody ketone donor substrates. B, Catalytic antibody aldehyde acceptor substrates.

resemble the hapten, and simple aliphatic aldehydes. Polyhydroxylated aldehydes, such as glyceraldehyde, glucose, and ribose, are not substrates, most likely because of the hydrophobic nature of the active site. In contrast to the natural aldolases, aromatic and α,β-unsaturated aldehydes are excellent substrates.

The stereochemistry of the addition is donor dependent. When acetone is used as the donor substrate, addition occurs from the *si* face of the carbonyl group; with hydroxyacetone, addition occurs from the *re* face. The stereoselectivity is generally quite high, with *ee* values greater than 99% commonly observed. As a general rule, high enantioselectivity is observed with acceptors having an sp^2 center in the α-position, and lower enantioselectivities are observed for α-position sp^3 centers.

The utility of catalytic antibodies was demonstrated with the antibody-catalyzed aldolase approach to the brevicomins sp[213] and the epothilones[214] (Fig. 14.1-39). Antibody 38C2 is commercially available and has recently been used as a catalyst to activate prodrugs[215]. Generic, drug-masking groups can be selectively removed by sequential retro-aldol and retro-Michael reactions catalyzed by 38C2 (Fig. 14.1-40). The antibody was also used in the enantioselective retro-aldol reaction of tertiary aldols containing heteroatom-substituted quaternary carbon centers[216]. This gave enantiomerically enriched tertiary aldols, most with *ee* values greater than 95%. Synthesis of enantiomerically pure tertiary aldols using the catalytic asymmetric aldol reaction with ketone acceptors represents a significant challenge. Compounds prepared in this study have been used in the synthesis of (+)-frontalin, the side chain of saframycin H, and mevalonolactone.

In order to increase the repertoire and efficiency of the aldol reaction further, and

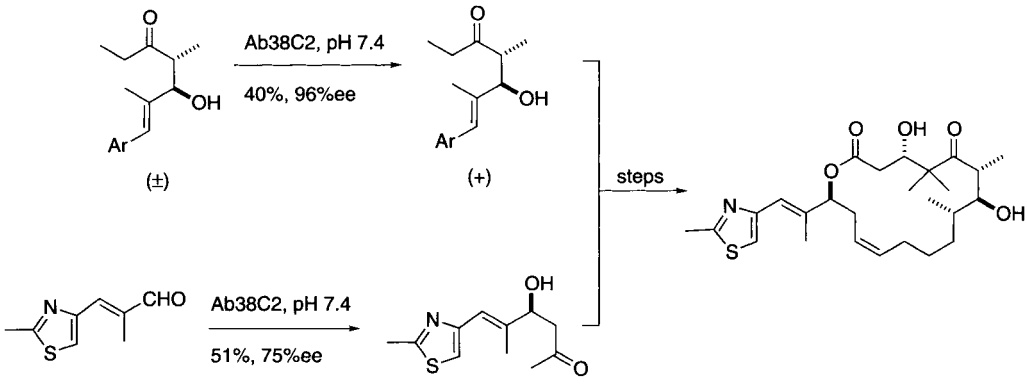


Figure 14.1-39. Use of catalytic antibody 38C2 for the preparation of epothilone intermediates.

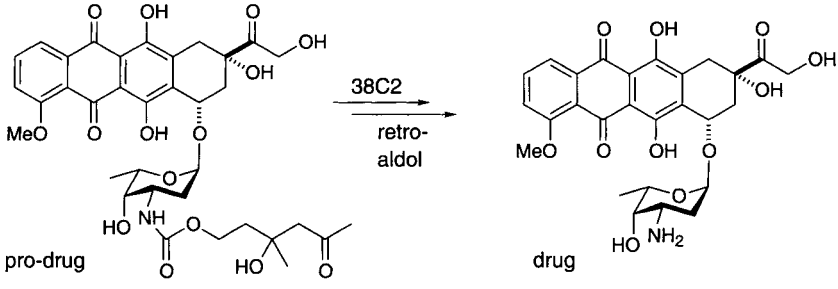


Figure 14.1-40. Retro-aldol reaction catalyzed by Ab 38C2 for the unmasking of pro-drugs.

to develop antibodies with complementary enantioselectivity, a β-diketone sulfone was employed as the hapten[217] (Fig. 14.1-41). The tetrahedral geometry of the sulfone moeity in this hapten mimics the rate-determining tetrahedral transition state of the C-C bond forming reaction. It is thus expected to facilitate nucleophilic attack of the enaminone intermediate on the acceptor aldehyde. It was indeed demonstrated that catalytic antibodies with broad reaction scope can be generated using this approach. In addition, antibody 93F3 was more efficient ($k_{cat} \sim 3 min^{-1}$) than and enantiocomplementary to 38C2, providing the unreacted (*S*)-aldol with >96% *ee*.

The mechanism-based approach to eliciting catalytic antibodies combined with the rapid, immune-selection process as illustrated in these studies provides a new and exciting direction for catalyst design and development.

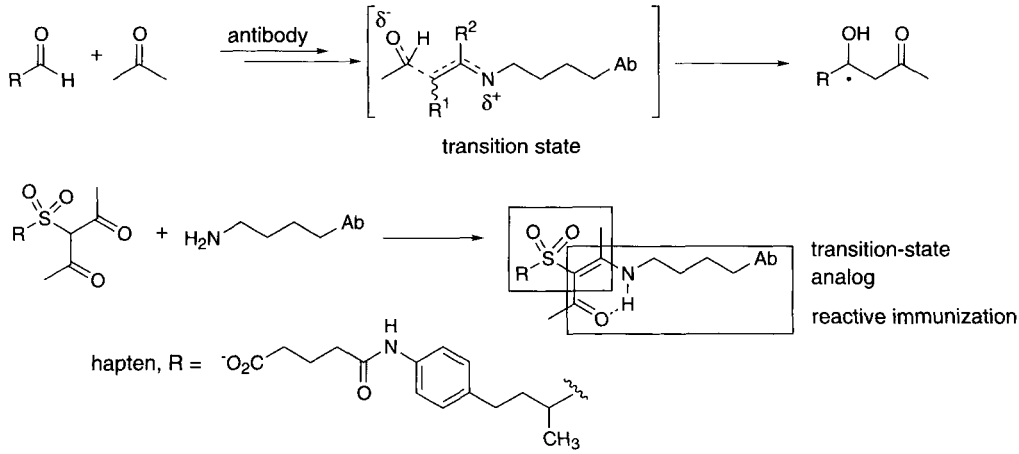


Figure 14.1-41. β-Diketone sulfone as hapten for reactive immunization.

14.2 Ketol and Aldol Transfer Reactions

14.2.1 Transketolase (TK) (E.C. 2.2.1.1)

TK is one of the enzymes involved in the oxidative pentose phosphate pathway, and requires the cofactors thiamine pyrophosphate (TPP)[219] and Mg^{2+} [218]. It reversibly transfers the C1-C2 ketol unit from D-xylulose 5-phosphate to D-ribose 5-phosphate, and generates D-sedoheptulose 7-phosphate and D-Gly 3-P. D-Erythrose 4-phosphate also functions as an acceptor of the ketol unit from D-xylulose 5-phosphate, to produce Fru 6-P and D-Gly 3-P (Fig. 14.2-1). TK from baker's yeast is commercially available, and the enzyme can also be isolated from spinach[220, 221]. TK from *E. coli* has been overexpressed and prepared on a large scale[222]. In ketol transfer reactions,

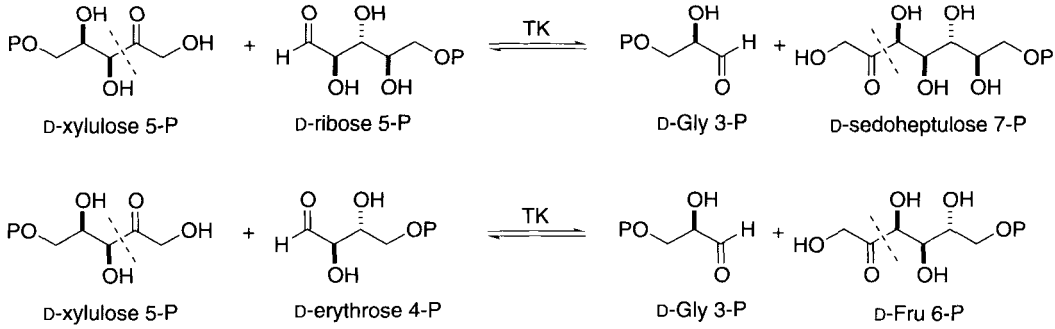


Figure 14.2-1. Ketol transfer reactions in the oxidative pentose phosphate pathway catalyzed by TK.

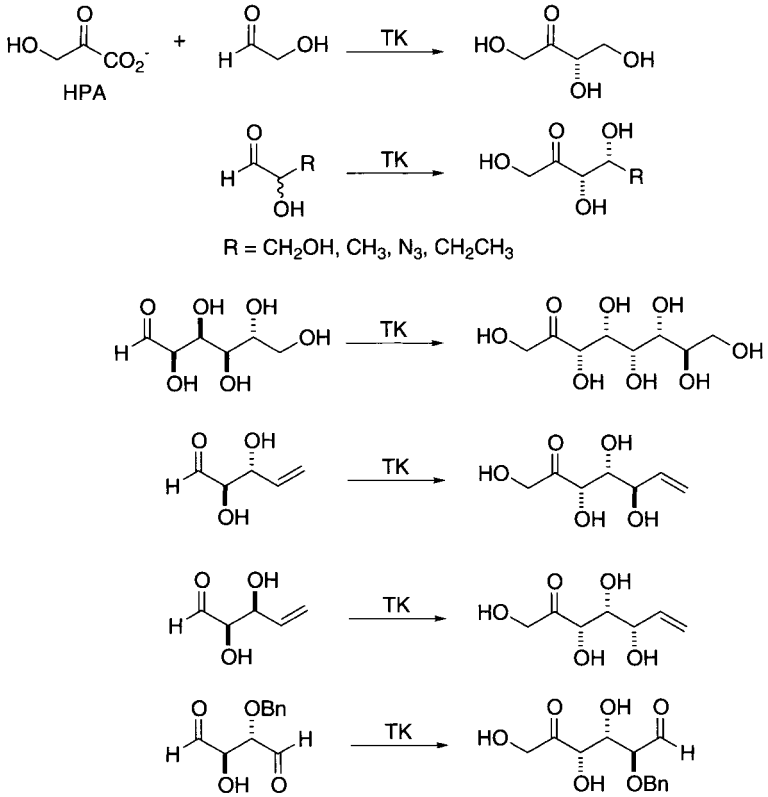


Figure 14.2-2. Acceptor substrate specificity of TK.

the enzyme isolated from yeast shows a higher diastereoselectivity (~ 100 %) [221] than that from spinach (~ 95 %), with the newly-formed hydroxymethine chiral center always possessing an (*S*)-configuration. TK also accepts β-hydroxypyruvic acid (HPA) as a ketol donor [223], and an efficient multi-enzyme synthesis of D-xylulose 5-phosphate employing FDP aldolase and *E. coli* transketolase has been reported [224]. The ketol unit is transferred to an aldose acceptor with an activity of 4 % compared with D-xylulose 5-phosphate [220]. This has been an invaluable discovery for the use of TK in synthesis, as the decarboxylation of HPA and subsequent loss of carbon dioxide, render the overall condensation reaction irreversible. A wide range of aldehydes are ketol acceptors, including aliphatic, α,β-unsaturated, aromatic, and heterocyclic aldehydes, although some are relatively poor substrates (Fig. 14.2-2) [225, 226]. The presence of a hydroxyl or an oxygen atom at C2 and/or C3 has a positive effect on the rate, while steric hindrance near the aldehyde exerts a negative effect. β-D-Hydroxy aldehydes (and not L-) are substrates, producing vicinal diol products of D-*threo* configuration [225, 227]. This allows efficient resolution of aldehydes epimeric at C2 by transketolase. The enzyme appears to have no preference for configuration beyond C2.

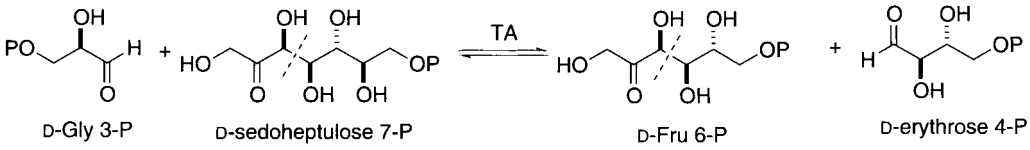


Figure 14.2-3. Aldol transfer reaction in the oxidative pentose phosphate pathway catalyzed by TA.

Figure 14.2-4. Multi-enzyme synthesis of D-Fru from starch.

TK has been used to catalyze the key step in the synthesis of the naturally occurring beetle pheromone (+)-*exo*-brevicomin[228] and the azasugar 1,4-dideoxy-1,4-imino-D-arabinitol[39]. Both syntheses involve the condensation of HPA with racemic 2-hydroxyaldehydes, whereby the ketol unit is diasteroselectively transferred to only the D-enantiomer of the aldehyde. In addition, transketolase has been employed in the synthesis of complex heptuloses[229], fructose analogs[230], and other sugars[231]. Erythrulose has been continuously produced through transketolase-catalysis in a membrane reactor[232].

14.2.2
Transaldolase (TA) (E.C. 2.2.1.2)

TA is also an enzyme of the oxidative pentose phosphate pathway[218]. It catalyzes the transfer of the C1-C3 aldol unit from D-sedoheptulose 7-phosphate to D-Gly 3-P, and produces D-Fru 6-P and D-erythrose 4-phosphate (Fig. 14.2-3). TA forms a Schiff base intermediate and does not require any co-factors. This enzyme is commercially available, and was used in a multi-enzyme synthesis of D-Fru from starch (Fig. 14.2-4)[233]. Here, it accomplished transfer of an aldol moiety from Fru 6-P to D-glyceraldehyde, and formed D-Gly 3-P and D-Fru.

14.3
Acyloin Condensation

Acyloin condensation catalyzed by yeast was first observed in the early part of the twentieth century[234, 235]. Yeast-catalyzed acyloin condensations between acetaldehyde and benzaldehyde derivatives have since been reported, giving products with a (*R*)-configuration in all cases (Fig. 14.3-1)[236, 237]. The acyloin formed from benzaldehyde alone has been used in the industrial manufacture of (–)-ephedrine[238]. It is

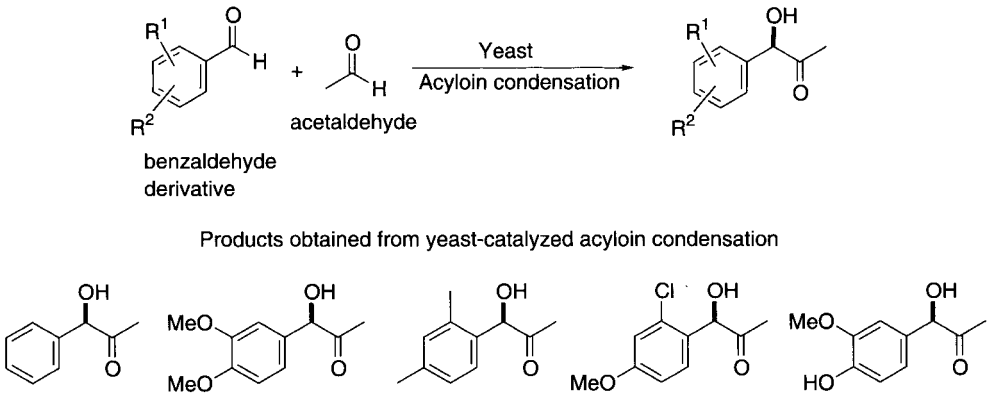


Figure 14.3-1. Acyloin condensation between acetaldehyde and benzaldehyde derivatives catalyzed by yeast.

probably the enzyme α-carboxylase (E.C. 4.1.1.1) that is responsible for catalyzing the acyloin reactions, as the carboxylase-catalyzed reaction of pyruvate and benzaldehyde in the presence of the cofactor thiamine pyrophosphate gives the corresponding acyloin product[239]. Pyruvate decarboxylase in highly purified[240] or partially purified forms[241] catalyzes acyloin condensation to give products of the (*R*)-configuration.

14.4
C-C Bond Forming Reactions Involving AcetylCoA

Enzymatic reactions which utilize coenzyme A thioesters as substrates are involved in the biosynthesis of steroids, terpenoids, macrolides, fatty acids, and other natural products. Owing to the high cost of CoA, these enzymes can only be practically used in organic synthesis if the CoA thioesters can be recycled. AcetylCoA can be efficiently regenerated by using one of several enzymatic systems[242–244]. Phosphotransacetylase (E.C. 2.3.1.8)/acetylphosphate, carnitine acetyltransferase (E.C. 2.3.1.7)/acetylcarnitine, and acetylCoA synthetase (E.C. 6.2.1.1)/ATP have all been employed for this purpose. These enzymatic recycling systems have been coupled to the synthesis of citric acid catalyzed by citrate synthetase. An interesting non-enzymatic regeneration of acetylCoA utilizes phase transfer catalysts in a two-phase aqueous-organic system (Fig. 14.4-1)[245]. Citric acid was efficiently prepared using this procedure, and this method also offers the potential to prepare many different acylCoA derivatives for use as substrates of CoA-dependent enzymes.

AcetylCoA is also involved in the biosynthesis of poly-β-hydroxybutyrate (Fig. 14.4-2, $x = 0$). Many whole cell systems have been used to synthesize this polymer and other interesting materials in this class[246]. For example, copolymers consisting of (*R*)-3-hydroxybutyl and (*R*)-3-hydroxyvaleryl units (Fig. 14.4-2, $x = 0$ and 1, respectively) were prepared by feeding propionate to whole cells of *A. eutrophus*[246].

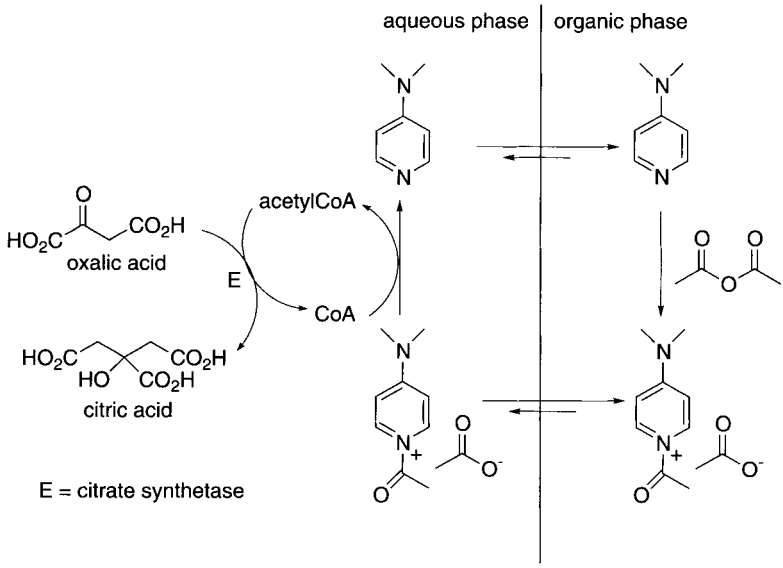


Figure 14.4-1. Chemical regeneration of acetylCoA using a phase transfer catalyst.

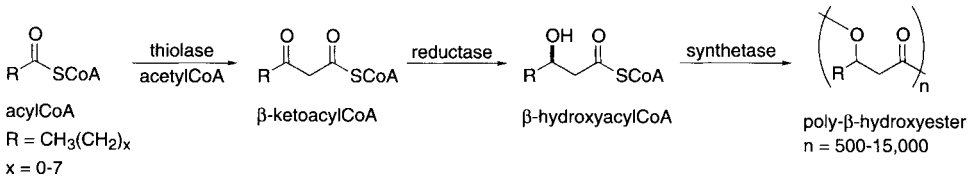


Figure 14.4-2. Enzyme-catalyzed reactions involved in the whole-cell synthesis of poly-β-hydroxyesters.

AcetoacetylCoA thiolase (E.C. 2.3.1.9), acetoacetylCoA reductase (E.C. 1.1.1.36), and polyhydroxybutyrate synthetase[247] are the enzymes involved in polyester synthesis. AcetoacetylCoA thiolase catalyzes the head-to-tail Claisen condensation of two acetylCoA molecules. In this reaction, the active site cysteine attacks acetylCoA to form a thioester enzyme intermediate, which then reacts with the enolate derived from enzymatic deprotonation of the other acetylCoA. Mechanistic studies have been performed on this enzyme from *Zooglea ramigera*, which has been cloned and overexpressed[247]. It has been established that the thiolase will form acyl enzyme intermediates with a number of acylCoA substrates, but will only accept acetylCoA as the nucleophile. After subsequent reduction, this results in all polymer units possessing a β-hydroxy group. These polymers are also useful sources of (*R*)-β-hydroxy acids[248].

14.5
Isoprenoid and Steroid Synthesis

Enzymes involved in the biosynthesis of isoprenoids and steroids have been used in organic synthesis[249]. 2,3-Oxidosqualene lanosterol cyclase was used to synthesize a number of lanosterol analogs (Fig. 14.5-1)[250–253]. When using an enzyme suspension from baker's yeast containing this cyclase, ultrasonic irradiation proved very effective in promoting catalysis[251, 252]. An interesting property of lanosterol cyclase was utilized during the synthesis of C30 functionalized lanosterols, whereby the enzyme rearranged a vinyl group rather than the usual hydrogen or methyl group[251]. This product was subsequently converted into (+)-30-hydroxylanosterol and the corresponding aldehyde. These compounds are natural receptor-mediated feedback inhibitors of HMG-CoA reductase, and therefore are of interest in the design of hypocholesteremic drugs[254].

Both enantiomers of 4-methyldihomofarnesol were synthesized using farnesyl diphosphate synthetase from pig liver, the (*S*)-enantiomer being a precursor of juvenile hormone (Fig. 14.5-2)[255]. Alkyl group homologs of isopentenyl diphosphate have also been examined as substrates for farnesyl diphosphate synthase[256].

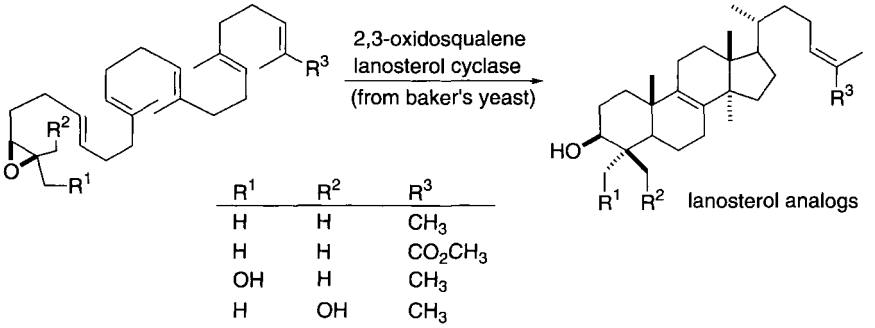


Figure 14.5-1. Synthesis of lanosterol analogues using 2,3-oxidosqualene lanosterol cyclase.

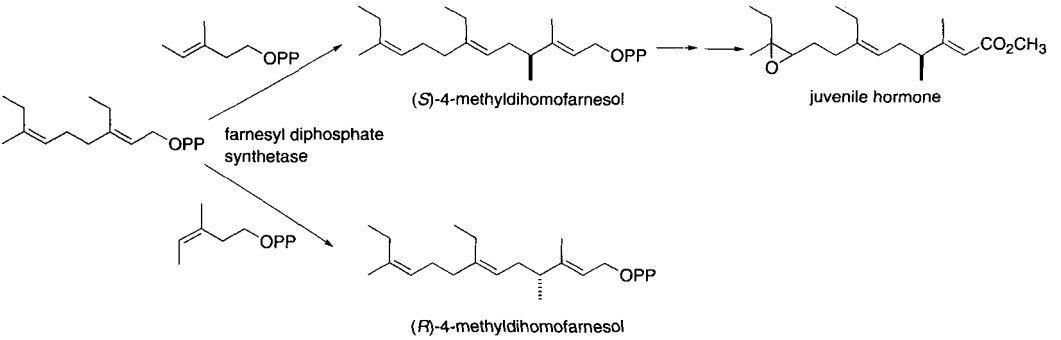


Figure 14.5-2. Synthesis of both enantiomers of 4-methyldihomofarnesol using farnesyl diphosphate synthetase.

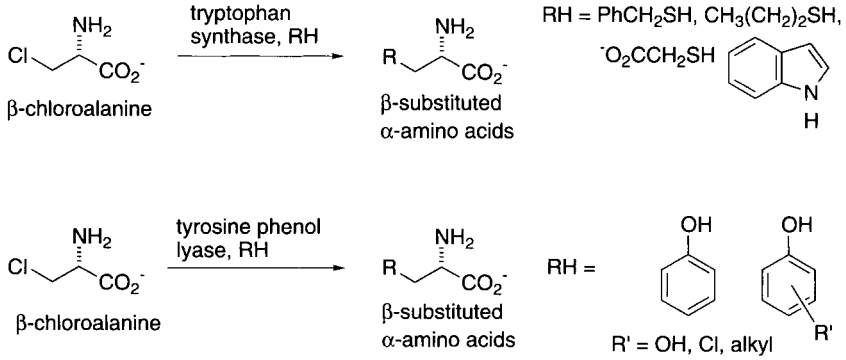


Figure 14.6-1. Synthesis of β-substituted α-amino acids from β-chloroalanine using tryptophan synthase and tyrosine phenol lyase.

14.6 β-Replacement of Chloroalanine

Methods have been developed for the synthesis of unnatural amino acids using pyridoxal phosphate-dependent enzymes [257]. These enzymes usually catalyze transaminations, α,β-eliminations, α,γ-eliminations, and decarboxylations of amino acids. However, using β-chloroalanine as a substrate, unusual amino acids are produced by β-replacement. Tryptophan synthase (E.C. 4.2.1.20) from *E. coli* catalyzes the formation of tryptophan and analogs. This enzyme has been employed to incorporate various heteroatoms into tryptophan, such as selenium [258], sulfur [259], nitrogen [260], chloride [261], and deuterium [262]. Notably, tryptophan synthase could be used to catalyze exchange of the α-proton from Asn, Glu, Ser, Ala, Phe, and Met as well as that of Trp [262]. Tyrosine phenol lyase (E.C. 4.1.99.2) (Fig. 14.6-1) has been utilized to synthesize tyrosine, DOPA, and methylated [263], fluorinated [264], and azido-tyrosine analogs [265].

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14.7
Enzymatic Synthesis of Cyanohydrins

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In the last decade, optically pure cyanohydrins (α-hydroxynitriles) have become a versatile source for the synthesis of a variety of chiral building blocks. Diverse methods for the enantioselective synthesis of cyanohydrins have been published and reviewed[1]. Besides enzyme catalyzed methods, hydrocyanation or silylcyanation of aldehydes or ketones controlled by chiral metal complexes or cyclic dipeptides, as well as diastereoselective hydrocyanation of chiral carbonyl compounds, have been applied with moderate success.

However, the most advantageous preparations of optically active cyanohydrins, with respect to the obtained enantioselectivities, are the enzymatically controlled approaches discussed in the present chapter. Two common enzyme systems are described and reviewed[1–16]: firstly, esterases or lipases, which have been employed

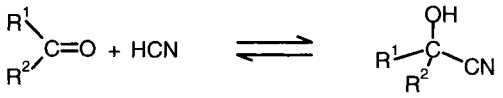

Figure 14.7-1. Cyanohydrin formation: R^1 = alkyl, cycloalkyl, aryl, heteroaryl; R^2 = H, alkyl.

for the resolution of racemic cyanohydrins or alkoxynitriles, and secondly, oxynitrilases – also known as hydroxynitrile lyases (HNLs), which catalyze the reversible formation of cyanohydrins (Fig. 14.7-1), using HCN and aldehydes or ketones.

About 3000 plant species are known to release HCN from their tissues, a process which is known as cyanogenesis[17, 18]. Storage compounds are cyanohydrins where the hydroxy function is glycosylated to a carbohydrate or protected as a fatty acid ester. The plant defence mechanism in the case of sugar compounds is a two-step reaction. Initially a glycosidase liberates the cyanohydrin moiety, which is cleaved either spontaneously by base catalysis or enzymatically by the action of oxynitrilases to release the corresponding carbonyl compound and HCN[19].

The application of an HNL was the subject of one of the earliest reports in the field of biocatalysis, namely the synthesis of mandelonitrile from benzaldehyde and hydrocyanic acid using a crude enzyme preparation obtained from almonds (termed "emulsin")[20]. However, little attention was paid to this discovery[21–23] until the 1960s, when this enzyme (E.C. 4.1.2.10) was isolated, characterized[24–26], and used for the preparation of enantiomerically enriched (*R*)-cyanohydrins from aromatic and aliphatic aldehydes[27–29]. The first examination of an (*S*)-oxynitrilase in millet seedlings (*Sorghum bicolor*, E.C. 4.1.2.11)[30–33] revealed that this enzyme only accepts aromatic substrates. At this time, the best enantiomeric excess obtained was 87% for the formation of (*R*)-mandelonitrile; other aldehydes gave even lower enantiomeric ratios.

14.7.1
The Oxynitrilases Commonly Used for Preparative Application

At present, the oxynitrilases from eleven cyanogenic plants (from six plant families) have been purified and characterised[9, 10].The properties of a selection of these are outlined in Table 14.7-1. The oxynitrilases E.C. 4.1.2.10 from Rosaceae (e.g. *Prunus* sp.) contain the cofactor FAD. However, the latter is not involved in redox reactions. Instead, it seems to have a structure-stabilizing effect, and its presence might be explained on evolutionary grounds[34–36]. Some of these enzymes are glycosylated and most of them are constructed from several subunits[14].

Recently the crystal structure of the oxynitrilase from *Hevea brasiliensis* (E.C. 4.1.2.39) was reported[37–41]. The enzyme was found to contain a large β-sheet which is surrounded by α-helices and a cap region on both sides. The active site is deeply buried inside the protein and connected to the surface by a narrow channel. Similar discoveries were published very recently for the (*S*)-HNL from *Manihot esculenta* (E.C. 4.1.2.39)[42, 43]. A big step forward, toward further applications of the *Prunus amygdalus* HNL, was achieved by the Kratky group by elucidating the crystal structure of this enzyme[44].

Table 14.7-1. Oxynitrilases available for organic synthesis.

Plant	Enzyme availability	Natural substrate	Substrate acceptance for syntheses	Stereo-selectivity
Prunus amygdalus	Almonds	(*R*)-Mandelonitrile	All R^1 and R^2	(*R*)
Linum usitatissimum	Flax seedlings overexpression	Acetone cyanohydrin (*R*)-2-Butanone cyanohydrin	Aliphatic aldehydes and ketones	(*R*)
Sorghum bicolor	Millet seedlings	(*S*)-4-Hydroxymandelonitrile	Aromatic aldehydes	(*S*)
Hevea brasiliensis	Rubber tree leaves overexpression	Acetone cyanohydrin	All R^1 and R^2	(*S*)
Manihot esculenta	Manioc leaves overexpression	Acetone cyanohydrin	All R^1 and R^2	(*S*)

Until quite recently, all HNLs had to be isolated from natural sources. To supply the industrial demand, enzymes from *Hevea brasiliensis*[45, 46] *Manihot esculenta*[47–49] and *Linum usitatissimum* (E.C. 4.1.2.37)[50–52] have been successfully overexpressed in several microorganisms. Presently, the (*S*)-cyanohydrin of 3-phenoxybenzaldehyde is used as an intermediate for various pyrethroid type insecticides; this reaction is catalyzed by overexpressed (*S*)-HNL from *H. brasiliensis* and the cyanohydrin is produced on the hundred ton per year scale[53].

In contrast to the HNLs from *H. brasiliensis* and *M. esculenta*, where aliphatic and aromatic aldehydes or ketones function as substrates, the HNL from *Sorghum bicolor* only catalyzes the formation and cleavage of aromatic (*S*)-cyanohydrins[54–59]. The most convenient natural sources of enzymes yielding products with (*R*)-stereochemistry are almonds (*Prunus amygdalus*)[60] and almond meal[61]. In addition to *Linum usitatissimum*[62], other sources of (*R*)-HNL have also recently been reported[63, 64]. Concerning the substrate spectrum, the *P.amygdalus* HNL catalyzes the HCN addition to aliphatic and aromatic carbonyl moieties, the *L.usitatissimum* oxynitrilase accepts only aliphatic ketones or aldehydes[65].

14.7.2
Oxynitrilase Catalyzed Addition of HCN to Aldehydes

(R)-Hydroxynitrile lyases. For preparative applications, (*R*)-HNL from almonds has been extensively investigated. Brussee et al.[66, 67] showed that without enzyme purification a crude extract from almond meal in aqueous methanol using *in situ* HCN generation from a solution of KCN in an acetate buffer affords cyanohydrins in up to 93% *ee*. Apple meal, in the form of unpurified enzyme preparations, accepts sterically hindered aldehydes (e.g. pivalaldehyde) as substrates, leading to (*R*)-cyanohydrins with high enantiomeric purity (usually *ee* >90%)[63, 68]. A purified enzyme from *Prunus amygdalus* supported on cellulose using nonaqueous systems was employed for the first time by Effenberger and co-workers[69]. Optimal results were obtained by almost completely suppressing the non-enzymatic HCN addition

using ethyl acetate as solvent. In this manner enantiomeric purity could be improved. Besides crystalline cellulose (Avicel), other hydrophobic enzyme immobilization systems such as Celite were used[70, 71]. Utilizing the natural support, unpurified almond meal in organic solvents with small amounts of aqueous phase (4%), provides products with *ees* of up to 99%[61, 68, 72–75]. Similar results were achieved with so-called "microaqueous systems"[76]. In order to reduce the amount of racemic cyanohydrin produced by chemical conversion, low concentrations of HCN were used by employing a relatively safe and convenient source of this reagent: acetone cyanohydrin[72, 73, 77–79]. Kanerva has developed a method where HCN diffuses into the reaction mixture from a second flask[74]. Wandrey used an enzyme membrane reactor for the continuous production of product employing an (*R*)-HNL. In a production run the volumetric yield was increased to 2400 g (*R*)-mandelonitrile/L × day with a residence time of just 3.8 min. The enzyme consumption was 17 000 U/kg product[80]. Applying a biphasic system a second industrial scale procedure was developed[81]. Based on these findings, four parameters (pH, concentration of HCN and benzaldehyde, temperature) were optimized to obtain a throughput of 6700 g (*R*)-mandelonitrile/L × day. A novel synthesis of (*R*)-cyanohydrins was described based on the use of cross-linked and subsequently polyvinyl alcohol-entrapped (*R*)-oxynitrilases. These immobilized lens-shaped biocatalysts have a well-defined macroscopic size in the mm range, show no catalyst leaching and can also be efficiently recycled. Furthermore, this immobilization method is cheap, and the entrapped (*R*)-oxynitrilases gave similar results to those using free enzymes. Accordingly, (*R*)-cyanohydrins were obtained in good yields and with high enantioselectivities of up to *ee* > 99%[82].

Some substrates, e.g. acrolein, gave only low optical purity with the *P. amygdalus* HNL. The catalytic capability of (*R*)-specific HNL from *L. usitatissimum* for the preparation of aliphatic cyanohydrins was investigated[50, 51, 65] and gave encouraging results (*ee* of up to 99%).

(S)-Hydroxynitrile lyases. As already mentioned, the (*S*)-hydroxynitrile lyase from *Sorghum bicolor* adds HCN only to aromatic and heteroaromatic aldehydes. Initial investigations were performed on the natural substrate 4-hydroxybenzaldehyde, and rather promising results concerning the enantiomeric excess were found[83]. These results were confirmed and extended using a suspension of enzyme immobilized on Avicel cellulose[84, 85] or etiolated shoots of *S.bicolor*[86] in diisopropyl ether. The *Sorghum* enzyme was one of the first recombinant hydroxynitrile lyases[87], overexpressed in *Escherichia coli*. In parallel to this work the *H. brasiliensis* HNL was also overexpressed[45], giving access to sufficient quantities of this enzyme both on a preparative scale and for industrial use. To date only a few preparative applications for *Sorghum* HNL[74] are known because of the narrow substrate range.

A similarly broad substrate range to that for the (*R*)-HNL from *Prunus amygdalus* is revealed by the (*S*)-HNLs from *Manihot esculenta* and *Hevea brasiliensis* (E.C. 4.1.2.39). Detailed sequence studies have revealed high homologies between both enzymes (*M. esculenta*[47, 88]; *H. brasiliensis*[36, 45]). This result was confirmed by the crystal structures. The latter was solved for *H. brasiliensis* in Graz[39] and for the *M. esculenta* enzyme in Stuttgart[43]. Expectations that these enzymes would be similar

with respect to substrate specificity were established by experimental data from both groups.

The cyanoglycoside Linamarin was found in 1965 in the seeds of the rubber tree (*Hevea brasiliensis*)[89].Two decades later the corresponding hydroxynitrile lyase was described[90, 91]. Studies regarding the synthetic potential of this enzyme with respect to the preparation of optically pure cyanohydrins started with the wild type[13, 92–94]. As already mentioned groundbreaking results were obtained with the synthesis of the (*S*)-cyanohydrin of 3-phenoxybenzaldehyde, this being a precursor for some important synthetic pyrethroids[53, 95–97].

HNL from *Manihot esculenta Crantz* (termed E.C. 4.1.2.37 at this time because E.C. 4.1.2.39 was not created earlier than 1999[98]) was purified to homogeneity from young leaves of the cyanogenic tropical crop plant cassava in 1994[47]. First experiments demonstrated a broad substrate range, but only unsatisfactory optical purities were obtained[99]. The overexpression of the cloned *M. esculenta* HNL gene in *E. coli* increased the accessibility and specific activity of the biocatalyst as well as the *ee* of produced cyanohydrins[87].

A selection of substrates with typical enantioselectivities of the obtained cyanohydrins, from the respective HNLs, is shown in table 14.7-2.

14.7.3
HNL-Catalyzed Addition of Hydrogen Cyanide to Ketones

Preparative elaboration of (*R*)-cyanohydrins of ketones employing oxynitrilase from *Prunus amygdalus* was first investigated in organic solvents[107]. Alkyl methyl ketones were obtained in moderate yields and in high optical purity, whereas with alkyl ethyl ketones the chemical and optical yields were reported to be lower[108]. The alteration, working with almond meal instead of purified enzyme, resulted in an astonishingly high enantiomeric excess[68]. Similar results with 98% *ee* for the (*R*)-cyanohydrin of butyl methyl ketone, were obtained[109].

(*R*)-Oxynitrilase from *Linum usitatissimum* has been used for the synthesis of (*R*)-butan-2-one cyanohydrin on a preparative scale[65].

Concerning (*S*)-ketone cyanohydrins, impressive results were gained on aliphatic or aromatic ketones, e.g. acetophenone cyanohydrin. The latter was obtained using the oxynitrilase from *H. brasiliensis*. (40% conversion, 99% *ee*)[101] or *M. esculenta* HNL (87% conversion, 98% *ee*)[110].

In table 14.7.3. the results gained by HNL-catalyzed conversions of selected methyl ketones to the corresponding cyanohydrins are shown.

14.7.4
Transhydrocyanation

The transcyanation (exactly termed transhydrocyanation) of aromatic and aliphatic aldehydes with acetone cyanohydrin, catalyzed by (*R*)-oxynitrilase to give cyanohydrins (see Fig. 14.7-2.), was first performed in Tallahassee[77]. This innovative method avoids the use of free HCN as the cyanide source and is mostly accompanied

Table 14.7-2. Aldehydes R-CHO as substrates for oxynitrilase-catalyzed cyanohydrin formation.

R	HNL	Source	Conversion [%]	*ee* [%]	Reference
Ph	*R*	*P.a.*	100	99	[61]
	S	*S.b.*	97	97	[74]
	S	*H.b.*	96	99	[13]
	S	*M.e.*	100	98	[87]
(*E*)-PhCH=CH	*R*	*P.a.*	54	87	[100]
	S	*H.b.*	93	98	[101]
3-PhO(C_6H_4)	*R*	*P.a.*	99	98	[69]
	S	*S.b.*	93	96	[102]
	S	*H.b.*	99	99	[101]
$PhCH_2OCH_2$	*S*	H.b.	92	12	[13]
$PhCH_2$	*R*	*P.a.*	83	88	[77]
	S	*H.b.*	44	99	[94]
$PhCH_2CH_2$	*R*	*L.u.*	10	10	[51]
	S	*H.b.*	88	93	[94]
2-$CH_3O(C_6H_4)$	*R*	*P.a.*	65	96	[77]
	S	*H.b.*	61	77	[94]
3-$CH_3O(C_6H_4)$	*R*	*P.a.*	85	98	[103]
	S	*S.b.*	93	89	[102]
	S	*H.b.*	80	99	[94]
4-$CH_3O(C_6H_4)$	*R*	*P.a.*	47	99	[61]
	S	*S.b.*	54	71	[74]
	S	*H.b.*	49	95	[94]
	S	*M.e.*	82	98	[87]
2-furyl	*R*	*P.a.*	96	99 (*S*)*	[104]
	S	*S.b.*	80	80(*R*)*	[104]
	S	*H.b.*	95	98(*R*)*	[101]
3-furyl	*R*	*P.a.*	96	99	[104]
	S	*S.b.*	88	87	[104]
	S	*H.b.*	98	98	[101]
	S	*M.e.*	98	92	[87]
2-thienyl	*R*	*P.a.*	71	99 (*S*)*	[104]
	S	*S.b.*	64	91(*R*)*	[104]
	S	*H.b.*	98	99(*R*)*	[101]
	S	*M.e.*	85	96(*R*)*	[87]
3-thienyl	*R*	*P.a.*	95	99	[104]
	S	*S.b.*	95	98	[104]
	S	*H.b.*	49	99	[94]
	S	*M.e.*	98	98	[87]
CH_2=CH	*R*	*L.u.*	100	74	[51]
	S	*H.b.*	92	98	[101]
	S	*M.e.*	70	56	[87]
(*E*)-CH_3CH=CH	*R*	*P.a.*	99	98	[100]
	S	*H.b.*	80	86	[93]
	S	*M.e.*	100	92	[87]
(*E*)-$CH_3(CH_2)_4$CH=CH	*S*	*H.b.*	96	99	[101]

Table 14.7-2. (cont.).

R	HNL	Source	Conversion [%]	*ee* [%]	Reference
(*E*)-$CH_3(CH_2)_2CH{=}CH$	*S*	*H.b.*	46	95	[93]
	S	*M.e.*	82	97	[87]
(*Z*)-$CH_3(CH_2)_2CH{=}CH$	*S*	*H.b.*	35	80	[93]
$CH_3(CH_2)_2C{\equiv}C$	*S*	*H.b.*	88	80	[93]
3-cyclohexenyl	*R*	*P.a.*	86	55	[67]
	S	*H.b.*	87	99	[94]
cyclohexyl	*R*	*P.a.*	90	99	[105]
	S	*H.b.*	95	99	[13]
	S	*M.e.*	100	92	[87]
$CH_3(CH_2)_7$	*R*	*P.a.*	82	96	[106]
	S	*H.b.*	35	85	[92]
$CH_3(CH_2)_4$	*R*	*P.a.*	72	97	[63]
	S	*H.b.*	81	96	[13]
$CH_3(CH_2)_2$	*R*	*P.a.*	99	98	[15]
	R	*L.u.*	91	98	[51]
	S	*H.b.*	80	80	[92]
	S	*M.e.*	70	88	[87]
$(CH_3)_2CH$	*R*	*P.a.*	99	83	[72]
	R	*L.u.*	100	93	[51]
	S	*H.b.*	80	81	[92]
	S	*M.e.*	91	95	[87]
$(CH_3)_3C$	*R*	*P.a.*	58	92	[77]
	R	*L.u.*	100	89	[51]
	S	*H.b.*	80	67	[92]
	S	*M.e.*	80	94	[87]

* Change of product configuration owing to a priority replacement according CIP rules
Abbreviations: HNL, hydroxynitrile lyase; *P.a.*, *Prunus amygdalus*; *S.b.*, *Sorghum bicolor*; *H.b.*, *Hevea brasiliensis*; *M.e.*, *Manihot esculenta*; *L.u.*, *Linum usitatissimum*.

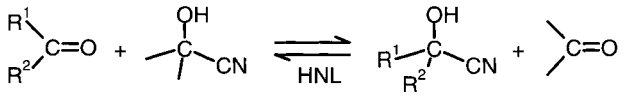

Figure 14.7-2. Principle of transhydrocyanation: R^1 = alkyl, cycloalkyl, aryl, heteroaryl; R^2 = H, alkyl.

by a slight decrease in *ee* compared to standard conditions. It was optimized in Turku[72] by comparing the feasibility of powdered almond meal as a catalyst to that of a purified enzyme preparation in an organic solvent.

The attempt to use racemic 2-methyl-2-hydroxyhexanenitrile as the cyanide donor was rewarded by obtaining aliphatic ω-bromo cyanohydrins from the corresponding aldehydes in 90–97 % *ee*[78]. As a biocatalyst, (*R*)-oxynitrilase was used.

Table 14.7-3. Methyl ketones R-CO-Me as substrate for oxynitrilase-catalyzed cyanohydrin formation.

R	HNL	Source	Conversion [%]	*ee* [%]	Reference
CH_3CH_2	*R*	*P.a.*	80	76	[107]
	R	*L.u.*	100	95	[51]
	S	*M.e.*	91	18	[87]
$CH_3(CH_2)_2$	*R*	*P.a.*	70	97	[107]
	R	*L.u.*	100	93	[51]
	S	*H.b.*	99	74	[101]
	S	*M.e.*	36	69	[87]
$CH_3(CH_2)_3$	*R*	*P.a.*	73	99	[68]
	S	*H.b.*	59	99	[101]
	S	*M.e.*	58	80	[87]
$(CH_3)_2CH$	*R*	*P.a.*	54	90	[107]
	S	*H.b.*	99	98	[101]
$(CH_3)_2CHCH_2$	*R*	*P.a.*	57	98	[107]
	S	*H.b.*	86	99	[101]
	S	*M.e.*	69	91	[87]
$(CH_3)_3C$	*S*	*H.b.*	49	78	[13]
	S	*M.e.*	81	28	[87]
Ph	*R*	*P.a.*	14	90	[68]
	S	*H.b.*	40	99	[101]
	S	*M.e.*	87	98	[110]
$PhCH_2$	*S*	H.b.	74	95	[13]

Abbreviations: HNL, hydroxynitrile lyase; *P.a.*, *Prunus amygdalus*; *L.u.*, *Linum usitatissimum*; *H.b.*, *Hevea brasiliensis*; *M.e.*, *Manihot esculenta*.

14.7.5
Experimental Techniques for HNL-Catalyzed Biotransformations

HNL catalysis in aqueous medium. Reaction in aqueous solution is performed with an appropriate acidic component and alkali cyanide for *in situ* development of the required HCN. The following procedure is a typical example[94].

To a stirred solution of 1 mmol aldehyde in 1.7 mL of 0.1 mol/L sodium citrate buffer (pH 4.0), 1 mL of a crude cytosolic extract of (*S*)-HNL from *Hevea brasiliensis* (100 IU/mL) was added and the mixture was cooled down to 0 °C. Subsequently, 2 mmol of potassium cyanide adjusted to pH 4.0 with cold 0.1 mol/L citric acid (17 mL) were added in one portion. After stirring for 1 h at 0–5 °C, the reaction mixture was extracted with methylene chloride (3 × 50 mL). The combined organic layers were dried over anhydrous sodium sulfate and the solvent was removed to give the crude cyanohydrin. This was then purified by column chromatography on silica gel using petroleum ether / ethyl acetate acidified with trace amounts of anhydrous HCl as the eluent.

HNL catalysis in organic medium. A significant advancement in cyanohydrin production was made by performing the transformation in organic solvents immiscible with water. It has been observed that there is virtually no spontaneous

chemical addition of HCN to the carbonyl moiety[48, 61, 69–71, 85, 105, 107, 111–114]. A representative protocol for cyanohydrin formation in organic solvents with immobilized oxynitrilase is the following[102].

A suspension of Avicel cellulose (0.5 g) in 0.05 mmol/L phosphate buffer (pH 4.5, 10 mL) containing ammonium sulfate (4.72 g) was stirred for 1 h, and a solution of (*S*)-HNL from *Sorghum bicolor* (50 μL, 1000 IU/mL, specific activity 70 IU/mg) was added. The mixture was stirred at room temperature for 10 min and filtered, and the immobilized enzyme was suspended in diisopropyl ether (10 mL). After addition of aldehyde (2 mmol) and dry liquid HCN (300 μL, 7.5 mmol), the mixture was stirred until all aldehyde had reacted. After removal of the immobilized enzyme, the filtrate was concentrated to yield the crude cyanohydrin.

HNL catalysis in biphasic medium. Biphasic solvent mixtures were reported employing (*R*)-oxynitrilase[81, 115] as well as (*S*)-HNL from *Hevea brasiliensis*[13, 101]. A typical procedure is as follows[81].

Freshly distilled benzaldehyde (37.1 g, 0.35 mmol), HCN (12.2 g, 0.45 mmol) and (*R*)-oxynitrilase (78 mg) were dissolved in 225 mL of methyl *t*-butyl ether (MTBE) and 250 mL of citrate buffer (50 mmol/L, pH 5.5) at 22 °C. After stirring for 20 min the MTBE layer was separated and the aqueous layer was extracted once with 25 mL of MTBE. The combined organic layers were dried over $MgSO_4$, filtered and concentrated under reduced pressure. Yield: 45.2 g (97 %), purity 98 %, *ee* 98 %. The aqueous layer was reused in a series of four consecutive experiments using the same amounts of reagents in the organic phase. A total of 185.5 g of benzaldehyde was converted into 226 g of (*R*)-mandelonitrile using 78 mg of (*R*)-oxynitrilase (0.035 wt%).

Transhydrocyanation for HCN generation. An alternative method of employing organic solvents that allows the safe use of HCN is transhydrocyanation [72, 73, 77–79, 116, 117]. An example of cyanohydrin formation using acetone cyanohydrin as the cyanide source is given in the following procedure[77].

To a solution of 120 mg (1 mmol) of phenylacetaldehyde and 110 mg (1.3 mmol) of acetone cyanohydrin in 11 mL of diethyl ether at 23 °C, 0.5 mL of (*R*)-oxynitrilase buffer solution (10 mg/mL, 0.4 mol/L acetate buffer, pH 5.0) was added. The mixture was stirred for 18 h at 23 °C and diluted with 50 mL of ether. The aqueous phase was extracted with 2 × 10 mL of ether and the combined organic phases were dried over anhydrous magnesium sulfate. Evaporation of solvent gave a pale amber liquid which was chromatographed on a flash silica gel column in 1 : 30 : 50 ethyl acetate / benzene / dichloromethane to afford 122 mg (83 %) of cyanohydrin, *ee* 88 %.

14.7.6
Resolution of Racemates

Oxynitrilase as catalyst. It is possible to treat a racemic cyanohydrin with a (*R*)- or (*S*)-HNL to decompose selectively one enantiomer of this mixture (exemplified in Fig. 14.7-3.). The (*R*)-HNL from *Prunus amygdalus* was used for the resolution of racemic cyanohydrins. Employing a biphasic system, namely citrate buffer/diisopropyl ether (40:1) at 39 °C, catalytic amounts of $PhNH_2$ and semicarbazide were added for

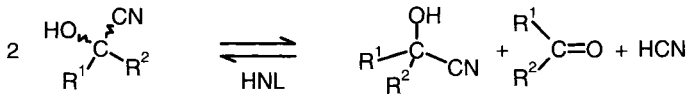

Figure 14.7-3. Enantioselective HNL catalyzed decomposition of racemic cyanohydrins: R^1 = alkyl, cycloalkyl, aryl, heteroaryl; R^2 = H, alkyl.

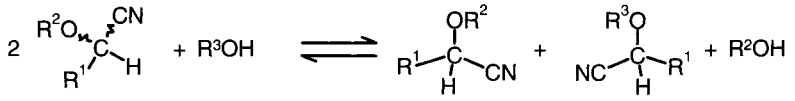

Figure 14.7-4. Lipase-catalyzed formation of optically enriched cyanohydrins: R^1 = alkyl, cycloalkyl, aryl, heteroaryl; R^2 = acyl; R^3 = H, acyl.

aldehyde capture. In this manner the (*S*)-cyanohydrin of 3-phenoxybenzaldehyde was obtained with 91% *ee* at 50% conversion[115].

Recently, almond meal was used for the resolution of *rac*-2-hydroxy-2-phenylpropanenitrile. Under the optimized conditions, (*S*)-2-hydroxy-2-phenylpropanenitrile, as the less reactive enantiomer, was obtained in 98–99% *ee* at approximately 50% conversion[118]. In a similar way the (*S*)-cyanohydrin was afforded from racemic 2-methyl-2-hydroxyhexanenitrile with *P. amygdalus* HNL in more than 90% *ee*[73, 78].

Esterase or lipase as catalyst. Application of hydrolytic enzymes is realized in three different systems: enzymatic hydrolysis or transesterification of racemic cyanohydrin esters (see Figure 14.7-4.) as well as enzymatic acylation of racemic cyanohydrins[119, 120].

A series of cyanohydrin acetates with an *e.e.* up to 98% has been prepared by enzymatic hydrolysis of their racemic acetates in the presence of an esterase from *Pseudomonas sp.*[137]. Lipoprotein lipase from *Pseudomonas sp.* catalysed irreversible transesterification using enol esters was applied to the resolution of different aromatic cyanohydrins[138, 139].

The enantioselective hydrolysis of the racemic acetate by *Arthrobacter* lipase gave the optically pure (*S*)-3-phenoxybenzaldehyde cyanohydrin. The unhydrolysed (*R*)-acetate was reracemised by heating with triethylamine and submitted again to enzymic hydrolysis[140]. In addition, the resolution of the racemic acetate ester of the cyanohydrin of 3-phenoxybenzaldehyde using a highly enantioselective lipase from *Pseudomonas sp.* was carried out recently with an *e.e.* of >96%[141]. Both the cyanohydrin esters and the free cyanohydrins (which are prone to racemization) can be isolated as enantiomers with high optical purity (*ee* 97%) on a preparative scale by the hydrolysis of the racemic butyrates with *Candida cylindracea* lipase and *Pseudomonas* sp. lipase[121].

A one-pot synthesis of optically active cyanohydrin acetates from aldehydes has been accomplished by lipase-catalyzed kinetic resolution coupled with *in situ* formation and racemization of cyanohydrins in an organic solvent. Racemic cyanohydrins, generated from aldehydes and acetone cyanohydrin in diisopropyl ether under the catalysis of basic anion-exchange resin, were acetylated stereoselectively by a lipase from *Pseudomonas cepacia* (Amano) with isopropenyl acetate as an acylating

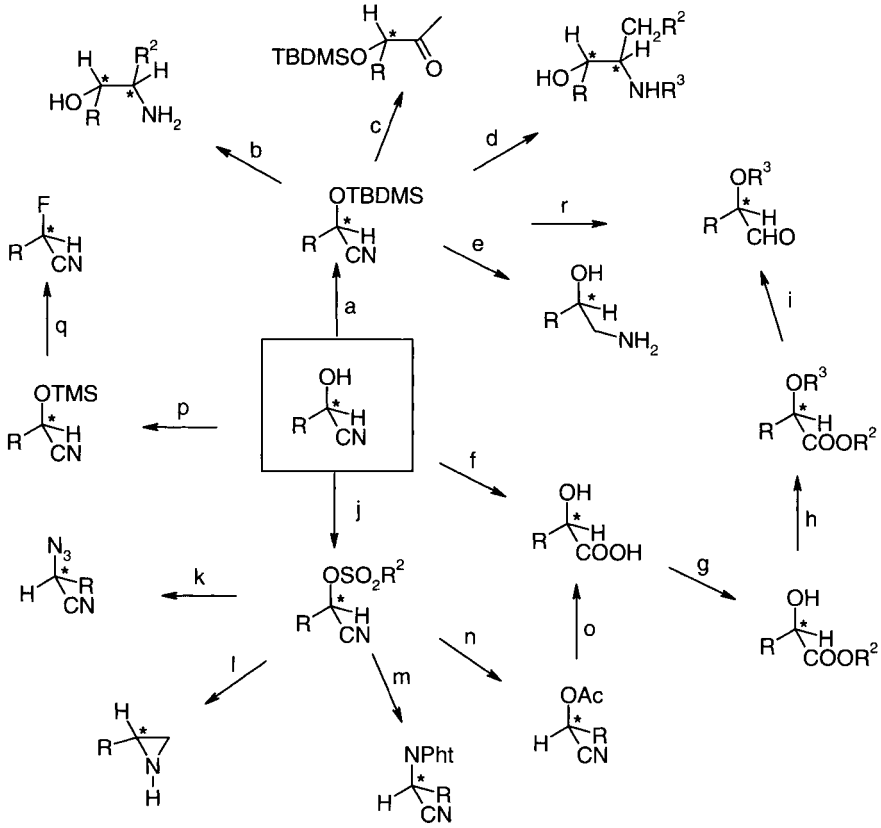

Figure 14.7-5. Follow-up reactions of optically pure cyanohydrins: a) TBDMSCl/imidazole[66, 67]; b) R^2MgX/ether, $NaBH_4$, H_3O^+[84, 125]; c) CH_3MgI/ether, H_3O^+[66]; d) R^2CH_2MgI/ether, MeOH, R^3NH_2, $NaBH_4$[126]; e) LAH[84]; f) H_3O^+[107]; g) $R^2OH/CHCl_3$/wolfatite[127]; h), R^3Cl/NaI/ CH_3CN/pyr/0 °C[127]; i) DIBALH/hexane/ −78 °C, conc. HCl/MeOH[127]; j) R^2SO_2Cl/pyr[102]; k) KN_3/DMF, (inversion)[128]; l) $LiAlH_4/Et_2O$/−80 °C, phosphate buffer pH 7.0/-70 °C[128]; m) potassium phtalimide/DMF, (inversion)[128]; n) KOAc/DMF/r.t., (inversion)[102, 128, 129]; o) conc. HCl/r.t. or lipase[102, 128, 129]; p) Me_3SiCl/pyr/Et_2O/0 to +25 °C[130]; q) diethylaminosulfur trifluoride (DAST)/CH_2Cl_2/-80 to +25 °C[130]; r) DIBALH/CH_2Cl_2/ −78 °C, 1N H_2SO_4[131].

reagent. The (*S*)-cyanohydrin was preferentially acetylated by the lipase, while the unreacted (*R*)-isomer was continuously racemized through reversible transhydrocyanation catalyzed by the resin. These processes consequently led to a one-pot conversion with up to 94% *ee* in 63–100% conversion yields[122, 123].

The *Pseudomonas aeruginosa* lipase (immobilised on hyflo Super-Cel) catalysed kinetic resolution of (*rac*)-2-(acetyloxy)-2-(pentafluorophenyl)acetonitrile gave enantiomerically pure cyanohydrin and its antipodal ester[142–144].

14.7.7
Follow-up Chemistry of Enantiopure Cyanohydrins

Optically pure cyanohydrins are important synthetic building blocks[1, 2, 4, 5, 9, 10, 15, 16, 124], as can be seen from Fig. 14.7-5. in selected examples. Both functional groups, the hydroxy and the cyanide moiety, can be easily converted into a large range of other chiral intermediates such as α-hydroxy acids and esters, α-hydroxy aldehydes and ketones, β-amino alcohols and α-fluorocyanides. These structural moieties are present in a large number of industrially valuable products such as drugs, agrochemicals, flavorings and fragrances.

14.7.8
Safe Handling of Cyanides

Hydrogen cyanide smells like bitter almonds, although many people cannot smell it at all[132]. Cyanide is a fast acting poison in the human body; it affects the ability of all cells to breathe. Severe breathing difficulties develop very rapidly when cyanide is swallowed, inhaled, or absorbed through the skin. Cyanide poisoning symptoms in the early stages include: general weakness, breathing difficulty, headache, nausea, giddiness, vomiting, the victims breath smelling like bitter almonds, and irritation of the nose, mouth, and throat. Hydrogen cyanide is liberated by the addition of acid to cyanide compounds.

The TLV (threshold limit value) for HCN is 11 mg/m^3 or 10 ppm[133]. These limits include the potential contribution of skin absorption to the overall exposure. Proper gloves should be worn when handling dry sodium cyanide. Rubber gloves and splash-proof goggles should also be worn when substantial amounts of sodium cyanide solution are used. All reaction equipment in which cyanides are used or produced should be placed in well-ventilated hoods, and it should be determined immediately whether anyone has been exposed to cyanide vapors or liquid splashing[134–136].

Vapor-detector tubes sensitive to 1 ppm of HCN are available commercially. The presence of free cyanide ion in aqueous solution may be detected by treating an aliquot of the sample with ferrous sulfate and an excess of sulfuric acid. A precipitate of Prussian blue indicates that free cyanide ion is present. More sophisticated for continuous warning is the use of electrochemical sensors for HCN detection.

Waste solutions containing cyanides treated with sodium hypochlorite are converted to harmless cyanate, which can be further processed to ammonia and carbon dioxide by addition of diluted sulfuric acid to pH 7. Surplus HCN gas can be neutralized by aqueous sodium hydroxide and then oxidized. Caution has to be advised with liquid hydrogen cyanide because bases including sodium hydroxide and sodium cyanide may initiate a violent polymerization[133].

Explosive hazards can occur on exposure of HCN to air in the presence of sources of ignition (flammable limits in air: 5.6–40% v/v) including heat (polymerizes explosively at 50–60 °C) and when HCN is stored for long periods of time.

14.7.9
Conclusions and Outlook

The enzymatic synthesis of enantiopure cyanohydrins has been brought to a high stage of development. Both (*R*)- and (*S*)-cyanohydrins are accessible for a broad variety of substrates in as a rule excellent yield and enantiopurity. Following recent progress in overexpression, HNLs are also available in quantities needed for industrial production. The procedures for safe handling of cyanides are well established so that they do not restrict the exploitation of HNLs.

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Enzyme Catalysis in Organic Synthesis
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15
Reduction Reactions

15.1
Reduction of Ketones

Kaoru Nakamura and Tomoko Matsuda

15.1.1
Introduction

15.1.1.1
Enzyme Classfication and Reaction Mechanism

Research on the asymmetric reduction of ketones by biocatalysis is expanding, and its practical applications to organic chemistry have resulted in success in the enantioselective synthesis of pharmaceuticals, agrochemicals and natural products[1–4]. It is attracting increasing attention because of the following advantages:

- providing a green and sustainable process (natural catalysis),
- high enantio-, regio- and chemo-selectivity compared with most man-made reagents and catalysts,
- achiral ketones can be transformed into the corresponding alcohols with 100% yield and 100% ee theoretically, whereas kinetic resolution of racemic substrates by hydrolytic enzymes such as lipases yields only 50% of products to achieve 100% ee.
- the resulting alcohol functionality can be easily transformed, without racemization, into other useful functional groups such as halides, thiols, amines, azides, *etc.*

Dehydrogenases, classified under E.C.1.1., are enzymes that catalyze reduction and oxidation of carbonyl groups and alcohols, respectively[5]. The natural substrates of the enzymes are alcohols such as ethanol, lactate, glycerol, *etc.* and the corresponding carbonyl compounds, but unnatural ketones can also be reduced enantioselectively. To exhibit catalytic activities, the enzymes require a coenzyme; most of the dehydrogenases use NADH or NADPH, and a few use flavin, pyrroloquinoline quinone, *etc.* The reaction mechanism of the dehydrogenase reduction is as follows:

Step 1 A holoenzyme (an enzyme with its coenzyme) binds a ketone.
Step 2 A hydride on the coenzyme is transferred to the ketone to produce an alcohol. (With concurrent oxidation of the coenzyme)
Step 3 The enzyme releases the product alcohol.
Step 4 The oxidized coenzyme is transformed back into the reduced form. (With concurrent oxidation of an auxiliary substrate)

There are four stereochemical patterns in the transfer of the hydride from the coenzyme, NAD(P)H, to the substrate (Step 2) as shown in Fig. 15-1. With E1 and E2 enzymes, the hydride attacks the *si*-face of the carbonyl group, whereas with E3 and E4 enzymes, the hydride attacks the *re*-face, which results in the formation of *R* and *S* alcohols, respectively. On the other hand, E1 and E3 enzymes transfer the pro-*R* hydride of the coenzymes, and E2 and E4 enzymes use the pro-*S* hydride. Examples of the E1–E3 enzymes are as follows:

E1: *Pseudomonas sp.* alcohol dehydrogenase[6]
Lactobacillus kefir alcohol dehydrogenase[7]
E2: *Geotrichum candidum* glycerol dehydrogenase[8–10]
Mucor javanicus dihydroxyacetone reductase[11]
E3: Yeast alcohol dehydrogenase[12]
Horse liver alcohol dehydrogenase[13–16]
Moraxella sp. alcohol dehydrogenase[17]

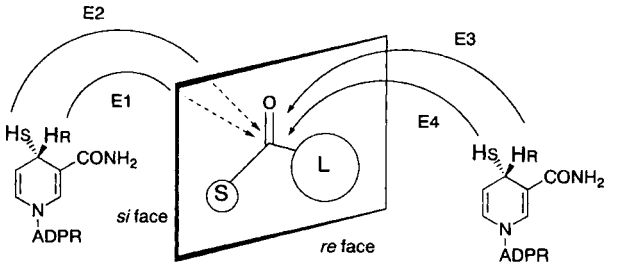


Figure 15-1. Stereochemistry of the hydride transfer from NAD(P)H to the carbonyl carbon on the substrate (S is a small group and L is a large group).

15.1.1.2
Coenzyme Regeneration

Reduction of the substrate accompanies the oxidation of the coenzyme (Step 2). Before the next cycle of the reduction of the main substrate can occur, the coenzyme has to be reduced (Step 4). Many methods for the regeneration of the reduced form of coenzyme [NAD(P)H] have been developed, so that only a catalytic amount of the coenzyme is required for the reaction. The coenzyme regeneration methods can be classified into two types:

- two-enzmye system: different enzymes reduce the substrate and $NAD(P)^+$,
- one-enzyme system: the substrate and $NAD(P)^+$ are both reduced by the same enzyme.

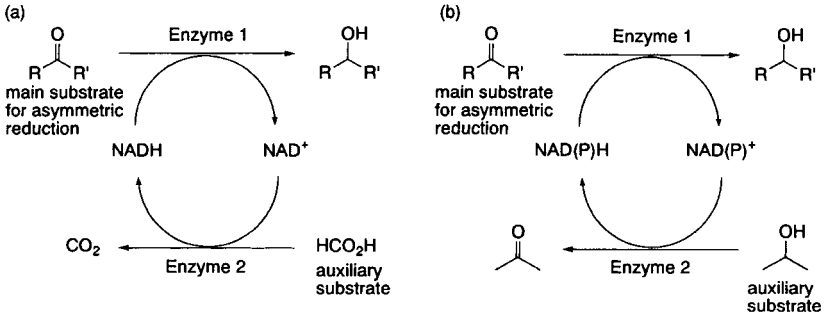


Figure 15-2. Regeneration of NAD(P)H: (a) Two-enzyme system using a formate dehydrogenase as an auxiliary enzyme and formic acid as an auxiliary substrate; Enzyme 1 = Enzyme for the reduction of the main substrate
Enzyme 2 = Formate dehydrogenase
(b) one-enzyme system using 2-propanol as an auxiliary substrate.
Enzyme 1 = Enzyme 2 For example alcohol dehydrogenase from *Thermoanaerobium brockii*[18, 19], *Pseudomonas sp.*[6], *Lactobacillus kefir*[7], and *Geotrichum candidum*[20, 21].

One of the examples of the two-enzyme system uses a formate dehydrogenase for the recycling of coenzyme [Fig. 15-2(a)][1, 3, 22–24]. It catalyzes oxidation of HCO_2H to CO_2 in order to drive the reduction of NAD^+ to NADH. The system is one of the most widely used due to the advantages such as: 1) the enzyme is commercially available, 2) CO_2 can be easily removed from the reaction, 3) formate is strongly reducing, therefore no back reaction occurs, and 4) CO_2 and HCO_2H are innocuous to enzymes. For example, the reduction of ethyl 4-chloro-3-oxobutanoate by a carbonyl reductase from *Rhodococcus eruthropolis* uses NAD^+/formate dehydrogenase as shown in Fig. 15-3[25]. As exemplified, the system is very useful for the recycling of NADH. However, it does not accept $NADP^+$, so it cannot be used for the direct reduction of $NADP^+$. For the reduction of $NADP^+$ using formate dehydrogenase, catalytic amounts of NAD^+ and $NAD(P)^+$ transhydrogenase are required. Changing the coenzyme specificity of a formate dehydrogenase using genetic methods is discussed in Sect. 15.1.3.7.

Two-enzyme systems using glucose dehydrogenase or glucose-6-phosphate dehydrogenase (commercially available enzymes) have also been widely employed[26–31].

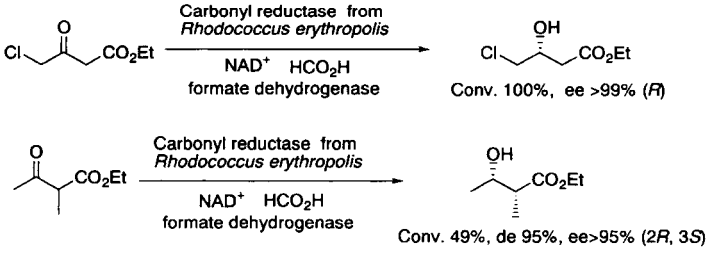


Figure 15-3. Examples of reduction using the formate/formate dehydrogenase NADH recycling system[25].

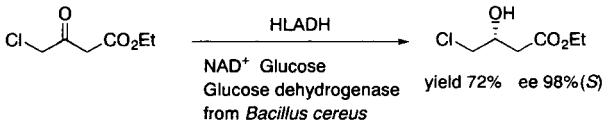


Figure 15-4. Example of reduction using glucose/glucose dehydrogenase NADH recycling system [26].

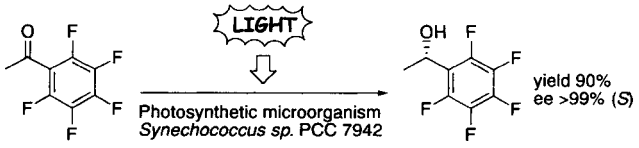


Figure 15-5. Utilization of light energy for an efficient reduction [32].

They oxidize glucose or glucose-6-phosphate to form gluconolactone or gluconolactone-6-phosphate, respectively, which is spontaneously hydrolyzed to give gluconic acid. Both NAD^+ and $NADP^+$ act as substrates for these enzymes. For example, a thermostable glucose dehydrogenase form *Bacillus cereus* was used to recycle NADH in the asymmetric reduction of ethyl 4-chloro-3-oxobutanoate by horse liver alcohol dehydrogenase (HLADH) as shown in Fig. 15-4 [26].

Another example of a two-enzyme system involves molecular hydrogen and a hydrogenase [1]. Hydrogenases catalyze the reduction of NAD^+ or other redox dyes by dihydrogen. The system is attractive because dihydrogen is inexpensive, strongly reducing and innocuous to enzymes and NAD(H), and no by-product is formed. However, a drawback is the extreme sensitivity of the hydrogenase enzymes to inactivation by dioxygen, preventing this system from being widely used.

To provide an environmentally friendly system, photochemical methods have been developed, which utilize light energy for the regeneration of NAD(P)H [1, 32, 33]. Recently, the use of cyanobacterium, a photosynthetic biocatalyst, for the reduction was reported where the effective reduction occurred under illumination (Fig. 15-5) [32]. When a photosynthetic organisms is omitted, the addition of a photosensitizer is necessary. The methods utilize light energy to promote the transfer of an electron from a photosensitizer *via* an electron transport reagent to $NAD(P)^+$ [1].

One-enzyme recycling systems are also well developed. One of the most frequently utilized is the alcohol-alcohol dehydrogenase system as shown in Fig. 15-2(b). The system does not need an auxiliary enzyme, but an auxiliary substrate is necessary. Ethanol or 2-propanol is frequently used as an auxiliary substrate. For example, HLADH uses ethanol as shown in Fig. 15-6 [13–16] and *Thermoanaerobium brockii* [18, 19], *Pseudomonas sp.* [6], *Lactobacillus kefir* [7], and *Geotrichum candidum* [20, 21] alcohol dehydrogenases recycle NAD(P)H by employing an excess of 2-propanol. A detailed investigation of the type and amount of the auxiliary substrate needed by *G. candidum* revealed that it can use 2-alkanols from 2-propanol to 2-octanol (and cyclopentanol as well), and 15–20 equivalents of the supplementary alcohol are necessary to shift the equilibrium (between the oxidation and reduction) towards the reduction of the main substrate. Because a much higher concentration

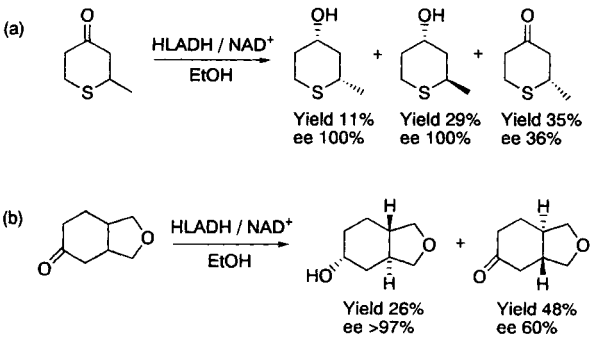


Figure 15-6. Reduction of heterocyclic ketones by HLADH using ethanol as an auxiliary substrate[15, 16].

of the auxiliary substrate to that of the main substrate is required, 2-propanol is deemed most suitable for synthetic purposes due to its high volatility.

Electrochemical regeneration of NAD(P)H represents another interesting method[34–36]. The system involves electron transfer from the electrode to the electron mediator such as methyl viologen or acetophenone *etc.*, then to the $NAD(P)^+$ (which is catalyzed by an electrocatalyst such as ferredoxin-$NADP^+$ reductase or alcohol dehydrogenase, *etc.*)[34]. Other methods involve the direct reduction of NAD^+ on the electrode[35]. Both one-enzyme systems and two-enzyme systems have been reported.

15.1.1.3
Form of the Biocatalysts: Isolated Enzyme vs. Whole Cell

Enzymes in a pure form, in a partially purified form, and in the whole cell can be used for organic synthesis, and each has advantages and disadvantages[3]. The proper choice of the form of the biocatalyst is important because it affects the enantio-, regio- and chemo-selectivities, the requirement (or not) of a coenzyme and an auxiliary enzyme, the ease of catalyst preparation and work up procedures, *etc.* as shown in Table 15-1.

The most widely used whole cell biocatalyst is bakers' yeast. Since it has many different kinds of enzymes, many kinds of substrate can thus be reduced, and various types of the reactions are expected. For example, β-keto esters, aromatic, aliphatic, cyclic and acyclic ketones can be reduced with high yield[1, 37–39]. Therefore, it is a versatile "all-round" reagent. However, since bakers' yeast contains many kinds of dehydrogenases, some of them may be *S* selective, while others are *R* selective, so that the enantioselectivities can be low to high depending on the substrate structure. Further degradation of the product may also be a problem, again associated with the fact that there are many kinds of enzymes in the cell.

Not only the enzymes but also the cellular components such as coenzymes and carbohydrates are conserved in the cell, which makes the whole cell processes favorable. For example, the addition of an expensive coenzyme and an auxiliary enzyme for coenzyme regeneration is not necessary, which makes the system simple and economical when comparing with the equivalent isolated enzyme process.

Table 15-1. The form of biocatalyst: whole cell vs. isolated enzyme.

Parameter	Whole Cell	Isolated Enzyme
Kinds of enzymes	Many	One
Kinds of reactions	Many	One
Regio- and enantioselectivity	Low to high	High
Coenzyme	Unnecessary	Necessary
Catalyst preparation	Easy	Difficult
Work up	Difficult	Easy
Example	Bakers' yeast	Horse liver alcohol dehydrogenase

However, the product isolation may be complicated due to large amounts of biomass and metabolites.

On the other hand, isolated enzyme processes also have many advantages. The problem associated with the product isolation and overmetabolism can be avoided using an isolated enzyme. More importantly, chemo-, regio-, and enantioselectivities of isolated enzyme systems are usually higher than that of whole cell processes because two competing enzymes with different stereoselectivities are not present. One of the most widely used isolated enzymes is horse liver alcohol dehydrogenase (HLADH) which reduces, for example, S-heterocyclic ketones to give the corresponding tetrahydrothiopyran-4-ol with 100% ee [Fig. 15-6(a)][15]. However, when the selectivity is so high, the substrate specificity is not wide; thus HLADH can reduce cyclic ketones with excellent enantioselectivity but cannot reduce acyclic ketones.

Another advantage of the isolated enzyme system is that the reaction pathway can be understood and predictions made. For example, for HLADH, the crystal structure[40–42] and the active site (diamond lattice) model[13, 14] are available to understand the reduction, whereas, in a whole cell process, even the catalytic species itself may not be clear.

In summary, whole cell and isolated enzyme biocatalysts both have various advantages and disadvantages. Using a recombinant yeast having the gene of a requisite enzyme is the way to access a single predominant enzyme in a microorganisms, a strategy which will be further discussed in Sect. 15.1.3.2.

15.1.1.4
Origin of Enzymes

Enzymes from various sources have been used for asymmetric reductions in organic synthesis. Microorganisms are the most important sources. There are a huge number of species (mostly in soil), containing a variety of enzymes. Commercially available microbial dehydrogenases are alcohol dehydrogenases from yeast, *Thermoanaerobium brockii* (TBADH), and the hydroxysteroid dehydrogenase from *Pseudomonas testosteroni*.

One of the most attractive kinds of microorganisms for organic synthesis is a thermophilic microorganism such as *Thermoanaerobium brockii*[18, 19], or *Thermoanaerobacter ethanolicus, etc.*[43–49]. The thermostability of the dehydrogenase en-

zymes from these microorganisms is very high; TBADH is stable even at 86 °C[18, 50] and an alcohol dehydrogenase from *Thermoanaerobacter ethanolicus* can be used at 50–60 °C[43, 47]. Since the enzymes with high thermostability usually have a high tolerance to organic solvent or substrates, the enzymes from thermophilic microorganisms are most suitable for organic synthesis.

Another interesting class of biocatalyst encompasses the photosynthetic microorganisms, the algae[32, 51]. Owing do the high growth rate, a large amount of the biomass for use as the biocatalyst is available. Importantly, such organisms can use light energy as power for coenzyme recycling as described in Sect. 15.1.1.2, so an environmentally friendly system can be constructed using them.

The second most widely studied source of enzymes are mammalian enzymes as exemplified by horse liver alcohol dehydrogenase (HLADH). Detailed investigations on this enzyme have been reviewed elsewhere[13, 14].

The third and least studies source is from plant cell cultures, which have only recently been used in biocatalysis[51–57]. Although the number of species available are much less than microorganisms, plants possess a much larger gene. More importantly since plants can effect photosynthesis, different types of enzymes exist in plants to those of microorganisms. Therefore, different enzymes which catalyze unique reactions with man-made substrates may be expected. Despite the strong possibility of the discovery of interesting enzymes, plant cell cultures have not been fully investigated for use in biocatalysis due to their relatively slow growth rate.

15.1.2
Stereochemical Control

15.1.2.1
Enantioselectivity of Reduction Reactions

The synthesis of enantiomerically pure compounds is becoming increasingly important for research and development in chemistry and biochemistry[58], especially in the pharmaceutical industry, as chiral drugs now represent close to one-third of all pharmaceutical sales world wide[59]. In most of the cases, one enantiomer is more effective as a drug than the other. The influence on the environment is also different between the enantiomers; different enantiomers of chiral pollutants in soils are preferentially degraded by microorganisms in various environments[60]. Therefore, synthetic methods exhibiting extremely high enantioselectivities are necessary.

The enzymatic reactions occurring in Nature involving natural substrates usually show very high enantioselectivities. On the other hand, with man-made substrates the enantioselectivity can also be high (> 99% ee) but this is not always the case as shown in Fig. 15-7. Low enantioselectivity results when the catalyst is a low selectivity enzyme [Fig. 15-7 (C)] and/or when there are more than two competing enzymes with different enantioselectivities [Fig. 15-7 (D)]. In case (C), either an enzyme or substrate has to be changed. On the other hand, in case (D), a change in a microorganism or substrate as well as a change in reaction conditions may be effective in improving the enantioselectivity. In case (D), by choosing the proper

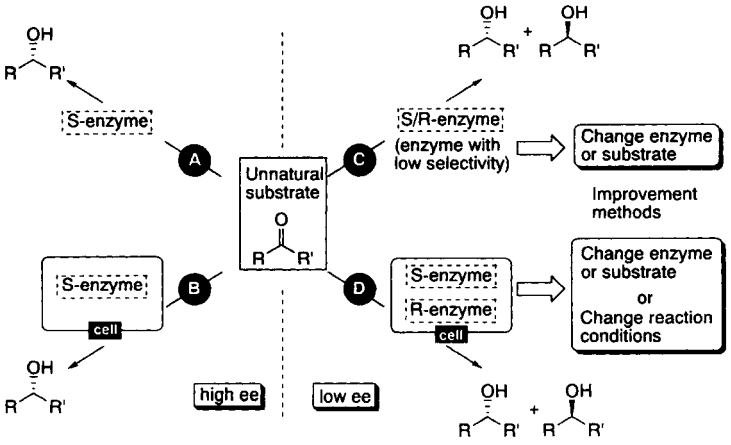


Figure 15-7. Enantioselectivity of the product and improvement methods.

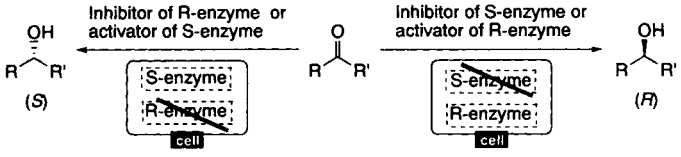


Figure 15-8. Synthesis of both enantiomers using one microorganism by choosing appropriate conditions.

conditions, both enantiomers can be synthesized by using only one microorganism; when a selective inhibitor for an *S*-directing enzyme or on *R*-directing enzyme is added to the reaction mixture, the *(R)*-alcohol or *(S)*-alcohol will be enantioselectively produced, respectively, as shown in Fig. 15-8.

15.1.2.2
Modification of the Substrate: Use of an "Enantiocontrolling" Group

The enantioselectivity of a biocatalytic reduction can be controlled by modifying the substrate because the enantioselectivity of the reduction reaction is profoundly affected by the structure of substrates. For example, in the reduction of 4-chloro-3-oxobutanoate by bakers' yeast, the ester moiety can be used to control the stereochemical course of the reduction[61–63]. When the ester moiety was smaller than a butyl group, then *(S)*-alcohols were obtained, and when it was larger than a pentyl group then *(R)*-alcohols were obtained as shown in Fig. 15-9.

After the reduction, the ester moiety can be exchanged easily without racemization, so both enantiomers of an equivalent synthetic building block are obtained using the same reaction system by changing an "enantiocontrolling" group, the ester moiety. The "enantiocontrolling" group can also be introduced into the keto esters at the α- or α'-positions. For example, sulfur functionalities such as methyl- and

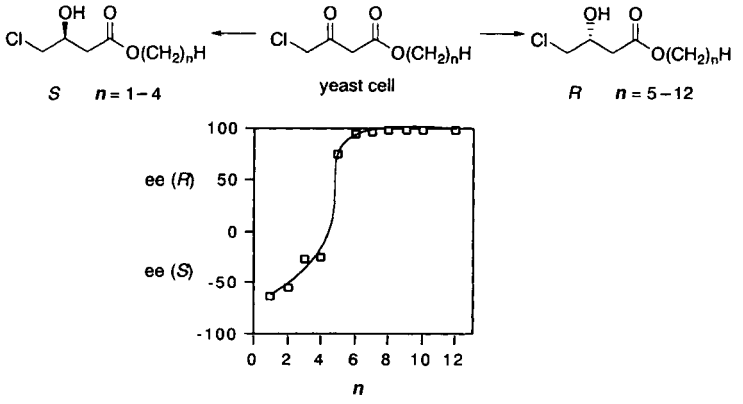


Figure 15-9. Stereochemical control on yeast-catalyzed reduction by changing the ester group[61–63].

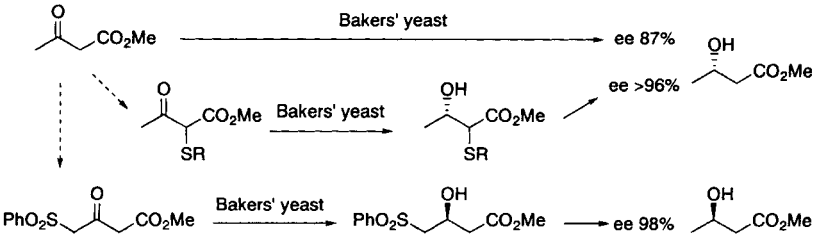


Figure 15-10. Stereochemical control on yeast-catalyzed reduction by introducing sulfur functionalities[64, 65].

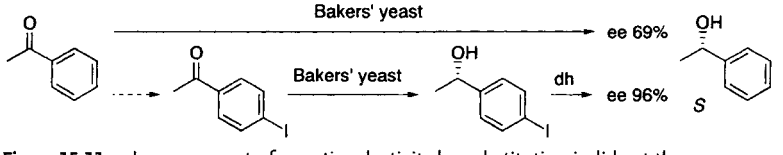


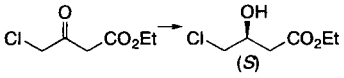
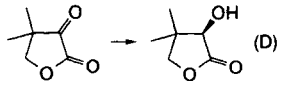
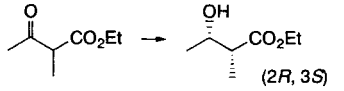
Figure 15-11. Improvement of enantioselectivity by substituting iodide at the *para* position; yeast reduction followed by dehalogenation (dh)[65].

phenylthio[64] and phenylsulfonyl[65] groups can be used to improve the enantioselectivities as shown in Fig. 15-10.

Other types of ketones can also be modified to improve the enantioselectivities, and various functionalities can be used to modify the substrate to produce the corresponding alcohol with higher enantioselectivities. For example, the reduction of acetophenone by yeast results in the formation of phenylethanol in 69% ee, whereas the reduction of *p*-iodoacetophenone followed by the dehalogenation results in a product of 96% ee (Fig. 15-11)[65].

As shown above, the substrate modification and "de"modification steps can be used to improve the enantioselectivity, although on the negative side the strategy may introduce extra steps into a synthetic route.

Table 15-2. Screening for the synthesis of important chiral building blocks.

Reactions	Microorganisms screened	Result	Reference
	400 yeasts	*Candida magnoliae* 90 g/L, 96.6% ee (99% ee after heat treatment)	67
	191 bacteria 59 actinomycetes 230 yeasts 81 molds 42 basidiomycetes	45 mg/mL stoichiometric yield *Rhodotorula minuta* IFO 0920: 86% ee *Candida parapsilosis* IFO 0708: 87% ee *Aspergillus niger* IFO 4415: 87% ee	68
	450 bacteria	*Klebsiella pneumoniae* IFO 3319 99% de, >99% ee, 99% yield (2 Kg in 200 L fermentor)	70

15.1.2.3

Screening of Microorganisms

Screening for a novel enzyme is a classical method and one of the most powerful tools available to find the system to convert a selected ketone into a desired alcohol[66–71]. It is possible to discover a suitable enzyme or microorganisms by the application of the newest screening and selection technologies that allows rapid identification of enzyme activities from diverse sources[66]. Enzyme sources for screening can be soil samples, commercial enzymes, culture sources, a clone bank, *etc.* From these sources, enzymes which are regularly expressed and enzymes which are not expressed in the original host can be tested to establish whether they are suitable for the transformation of certain substrates[66]. For example, 400 yeasts were screened for the reduction of ethyl 4-chloro-3-oxobutanoate, and *Candida magnoliae* was found to be the best one as shown in Table 15-2[67, 72, 73]. For the reduction of ketopantoyl lactone, various kinds of microorganisms were screened, and several microorganisms which produce D-(−)-pantoyl lactone stoichiometrically at a concentration of 45 mg mL^{-1} with high enantioselectivity were found[68]. For the reduction of ethyl 2-methyl-3-oxobutanoate, out of 450 bacteria, *Klebsiella pneumoniae* IFO 3319 and 4 other strains were found to give the corresponding (2*R*, 3*S*)-hydroxyesters with more than 98% de and > 99% ee[70].

Screening techniques have also been applied for the purpose of drug synthesis. For example, a key intermediate in the synthesis of the anti-asthma drug, Montelukast, was prepared from the ketone **1** by microbial transformation as shown in Fig. 15-12[71]. The biotransforming organism, *Microbacterium campoquemadoensis* (MB5614), was discovered as a result of an extensive screening programme.

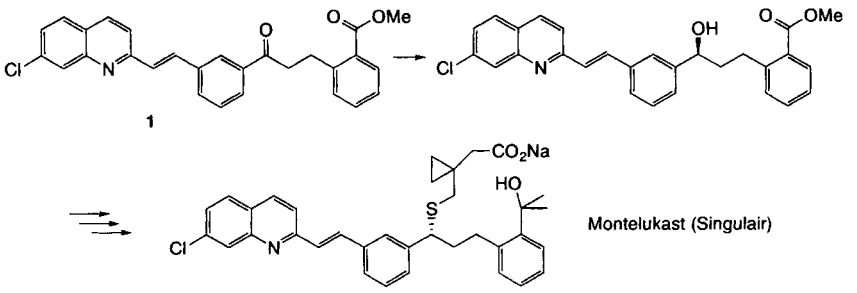
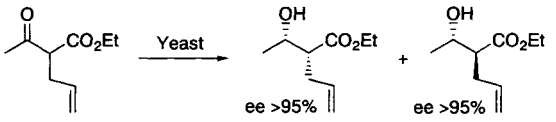


Figure 15-12. Reduction of a ketone by *Microbacterium campoquemadoensis* (MB5614) in a synthesis of the anti-asthma drug, Montelukast[71].

Table 15-3. Control on diastereoselectivity by heat treatment[74].



Yeast cell	*Syn* (%)	*Anti* (%)
No heat treatment	30	70
50 °C, 30 min	65	30
heat + inhibitor	96	4

15.1.2.4

Treatment of the Cell: Heat Treatment

Treatment of the cell before the reaction is sometimes an effective method of controlling the selectivity of some biocatalysts. When reducing with a whole cell and the selectivity is not as is desired due to the presence of plural enzymes with different selectivities, heat treatment of the cell to selectively deactivate one or more enzymes can change the selectivity of the reduction. For example, the diastereoselectivity in the yeast reduction of 2-allyl-3-oxobutanoate was changed from *anti*-selectivity to *syn*-selectivity by pre-treatment of the yeast before the reaction as shown in Table 15-3[74]. In this case, the diastereoselectivity is further improved to 96 : 4 by using an enzyme inhibitor.

Another example is the use of heat treatment as a supplement to the screening process. The enantioselectivity of the reduction of ethyl 4-chloro-3-oxobutanoate by *Candida magnoliae* was improved from 96.6 % ee *(S)* using untreated cells to 99 % ee *(S)* with heat treated cells[67].

15.1.2.5

Treatment of the Cell: Aging

When a whole cell system is used for a reduction, the substrate is usually added to the cultivation medium after a certain growth period, or to the mixture of the

medium and freshly harvested cells. However, when the mycelium of a local strain of *Geotrichum candidum* was not used immediately after growth, but filtered and preincubated by shaking in deionized water for 24 hours at 27 °C ("aged mycelium"), then used for the reduction of ethyl 3-oxobutanoate, the stereochemistry of the product alcohol was different from that obtained from the reduction using fresh mycelium[75–78]. When fresh mycelium was used, the enantioselectivity and the absolute configuration of the product shifted from *S* (26% ee) to *R* (58% ee) on raising the substrate concentration from 1 to 20 g L^{-1}. When aged mycelium was used, the absolute configuration was always *R* and showed constant enantioselectivity (ca. 50% ee) regardless of the substrate concentration, although the reduction proceeded at a slightly slower rate.

In the aging process, an *S*-forming activity, was lost, leaving unaffected either one low-specificity reducing enzyme with major *R*-forming activity, or several enzymes having opposite enantioselectivities but similar K_M values.

15.1.2.6
Treatment of the Cell: High Pressure Homogenization

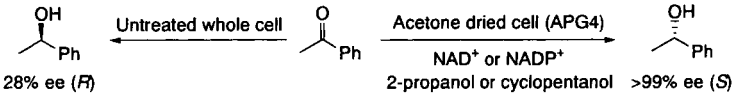
High pressure homogenization is a new technology in food processing. It was found that the same technology can be applied to effect the microbial reduction of chemical compounds[79]. The cell culture with substrate (such as acetophenone, 5-hexen-2-one, *etc.*) was poured into the high pressure homogenizer, and then it was incubated for 48 h and the enantioselectivity of the product was evaluated. During the process the reaction mixture was forced under pressure through a narrow gap where it was subjected to rapid acceleration [1 (blank experiment), 500, 1000, 1500 bar] after which it undergoes an extreme drop in pressure. Various strains of *Saccharomyces cerevisiae* and *Yarrowia lipolytica* are utilized in the reduction processes and higher enantioselectivities were generally achieved albeit in lower yields than the standard process.

15.1.2.7
Treatment of the Cell: Acetone Dehydration

A dried cell mass is often used as a biocatalyst for a reduction, since it can be stored for a long time and can be used whenever needed, without cultivation. One of the useful methods to dry the cell mass is acetone dehydration[80]. For example, the cells of *Geotrichum candidum* IFO 4597 were mixed with cold acetone (–20 °C) and the cells were collected by filtration[20, 21]. The procedure was repeated five times and then the cells were dried under reduced pressure. The dried cells (acetone powder of *G. candidum* IFO 4597: APG4) were obtained; they can be stored for a long time in the freezer.

The drying of the cell not only aids the preservation of the cell but also contributes to the stereochemical control as shown in Table 15-4. The reduction of acetophenone catalyzed by *G. candidum* IFO 4597 resulted in poor enantioselectivity [28% ee(*R*)]. When the form of the catalyst was changed from wet whole-cell to dried powdered-

Table 15-4. Acetone treatment of *Geotrichum candidum* for the improvement of enantioselectivity[20, 21].



Catalyst	Coenzyme	Additive	Yield (%)	ee (%)
Untreated whole cell	none	none	52	28(*R*)
Acetone dried cell (APG4)	none	none	0	–
Acetone dried cell (APG4)	NAD^+	2-propanol	89	>99(*S*)
Acetone dried cell (APG4)	NAD^+	cyclopentanol	97	>99(*S*)
Acetone dried cell (APG4)	$NADP^+$	cyclopentanol	86	>99(*S*)

cell (APG4), no reduction was observed, which would indicate the loss of the necessary coenzyme(s) and/or coenzyme regeneration system(s) during the treatment of the cells with acetone. Addition of coenzyme, NAD^+, did not have a significant effect on the yield. Addition of 2-propanol resulted in only a small increase in the yield, but a significant improvement in the enantioselectivity was observed. Surprisingly, addition of both NAD^+ and 2-propanol profoundly enhanced both chemical yield and enantiomeric excess. Addition of NADH, $NADP^+$ or NADPH instead of NAD^+ and addition of cyclopentanol instead of 2-propanol also gave enantiomerically pure alcohol in high yield.

The improvement in the enantioselectivity from 28% *(R)* to > 99% *(S)* was due to the suppression of every enzyme which reduces the substrate, followed by the stimulation of an *S*-directing enzyme by the addition of the coenzyme and an excess amount of 2-propanol, agents which push the equilibrium towards the reduction of the substrate.

It was confirmed, by separating the enzymes in the powder, that many *S*- and *R*-directing enzymes exist in the biocatalyst. The addition of coenzyme and cyclopentanol stimulates only one particula *S-enzyme* but not other *S-enzymes* and *R*-enzymes because the *specific* *S*-enzyme can oxidize cyclopentanol [concomitantly reducing $NAD(P)^+$], while other *S*- or *R*-enzymes cannot use cyclopentanol as effectively[81]. This is a very interesting case where the reduction with a cell initially having both *S*- and *R*-directing enzymes was modified and resulted in excellent *S* enantioselectivity.

15.1.2.8
Cultivation Conditions of the Cell

The dependence of enantioselectivity in microbial transformations on the cultivation conditions of the microorganisms has also been investigated[82–86]. The enzymes induced during the growth phase and during starvation are certainly different, therefore the enantioselectivity of the product may be different when two competing enzymes with different enantioselectivities catalyze the reduction. Since the enzyme

reducing the non-natural substrate is not usually known, cultivation conditions which induce the desired enzyme have to be found by trial and error.

For example, the effect of cultivation time and different carbon sources on the enantioselectivity of the reduction of sulcatone by some anaerobic bacteria has been investigated[83]. Another example is the investigation on the effect of the medium concentrations for cultivation of *Geotrichum candidum* IFO 4597 on the enantioselectivity of the reduction of acetophenone derivatives. The yield of *R*-alcohol (the minor enantiomer) increased with the medium concentration; therefore, the medium concentration was kept low, optimally to produce the *S*-enantiomer[82]. The effect of the aeration during cultivation on the enantioselectivity of bakers' yeast production of 3-hydroxyesters has also been reported[86]. Inducers such as a substrate analog may also induce the desired enzyme to improve the enantioselectivity.

15.1.2.9
Modification of Reaction Conditions: Incorporation of an Inhibitor

In the case of the observation of poor overall enantioselectivity due to the presence of two competing enzymes with different enantioselectivities, one of the most straightforward methods to improve the enantioselectivity is the use of the inhibitor of the unnecessary enzyme(s). Ethyl chloroacetate, methyl vinyl ketone, allyl alcohol, allyl bromide, sulfur compounds, Mg^{2+}, Ca^{2+}, *etc.* have been reported as inhibitors of enzymes in yeast[87–97].

For example, the low enantioselectivity in the yeast reduction of β-keto ester was improved by addition of ethyl chloroacetate or methyl vinyl ketone as described in Fig. 15-13. The enzymes inhibited and those not inhibited were identified by enzymatic studies using purified enzymes[97]. The mechanism of the inhibition is reported to be non-competitive.

These inhibitors were also used to improve the enenatioselective reduction of

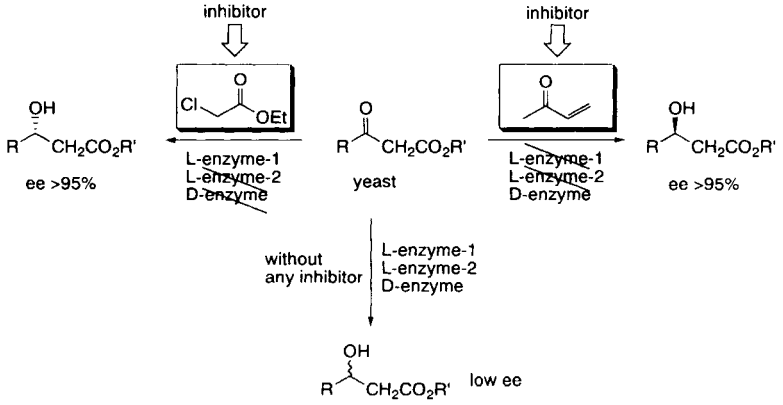


Figure 15-13. Improvement of the enantioselectivity by using an inhibitor of undesired enzymes[87, 88, 97].

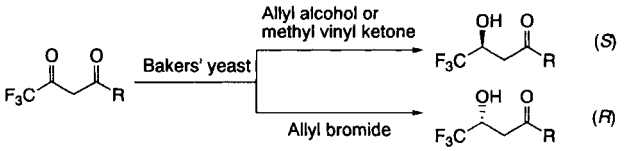


Figure 15-14. Stereochemical control using an inhibitor[89].

fluorinated diketones (Fig. 15-14). By applying a suitable inhibitor, both enantiomers of the alcohol can be obtained using only one kind of microorganism, namely bakers' yeast[89].

15.1.2.10
Modification of Reaction Conditions: Organic Solvent

Organic solvents have been used widely for esterifications and transesterifications using hydrolytic enzymes to shift the equilibrium towards esterification by avoiding hydrolysis. Organic solvents can also be used for reductions using dehydrogenases[98–109]. They can be used to control the overall enantioselectivity of the reduction, when there are more than two competing enzymes with different enantioselectivities, K_M and V_{max}.

Enzymatic reactions follow the Michaelis–Menten equation, therefore, the rate of the enzyme catalyzed reaction depends on the substrate concentration. When an organic solvent is introduced, most organic substrates usually dissolve in the organic phase, and the effective substrate concentration in the aqueous phase around the enzyme decreases. The change in substrate concentration by the addition of the organic phase causes the change in the enzyme species catalyzing the reduction. For example, as shown in Fig. 15-15, if the K_M for an *S*-directing enzyme is much smaller than that for an *R*-directing enzyme, and V_{max} for the *S*-directing enzyme is much smaller than that for the *R*-directing enzyme, then when the substrate concentration is low, the *S*-enzyme will dominate, whereas at high substrate concentration, the *R*-enzyme will dominate the biotransformation.

In fact, when the yeast reduction of ethyl 2-oxohexanoate was conducted in water,

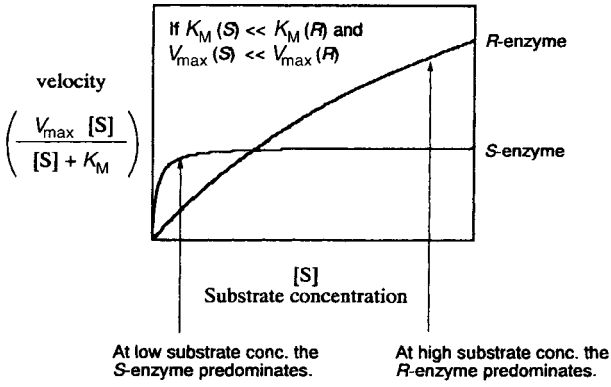


Figure 15-15. Effect of the substrate concentration on enantioselectivity of the reduction with the system having both an *S*-enzyme with small K_M and small V_{max} and an *R*-enzyme with large K_M and large V_{max}.

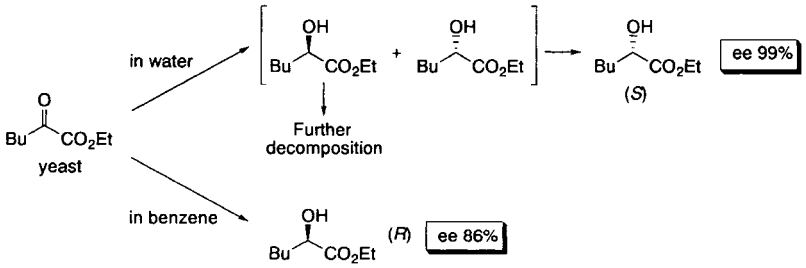


Figure 15-16. Stereochemical control by using an organic solvent.

Table 15-5. Mechanism of stereochemical control using benzene: kinetic parameters of yeast α-keto ester reductases (YKERs)[100, 110].

Enzyme	Enantioselectivity	K_M (mM)	k_{cat} (s^{-1})	V_{max} (U kg^{-1} yeast)
YKER-I	*R*	8.40	1.53	37.7
YKER-IV	*R*	0.142	4.59	41
YKER-V	*S*	5.72	27.8	649
YKER-VI	*R*	1.03	2.10	1774
YKER-VII	*S*	27.3	127	501

both *(R)*- and *(S)*-alcohols were produced and the *(S)*-alcohol was obtained as the major product as a result of the further enantioselective decomposition of the *(R)*-enantiomer (Fig. 15-16)[100, 109]. However, when the biotransformation was conducted in benzene, then the *(R)*-alcohol was formed selectively in high yield.

K_M and V_{max} for all enzymes existing in yeast and catalyzing the reduction were determined and it was found that an *R*-enzyme, YKER-IV, has a K_M which is smaller than other enzymes by an order of magnitude (Table 15-5), and, therefore, predominantly catalyzes the reduction in benzene[100, 110, 111].

15.1.2.11
Modification of Reaction Conditions: Use of a Supercritical Solvent

Supercritical fluids, materials above their critical pressure and critical temperature (Fig. 15-17), have been attracting attention as solvents with the advantages of gas-like low viscosities and high diffusivities coupled with their liquid-like solubilizing power[112]. Supercritical carbon dioxide ($scCO_2$) has the added benefit of an environmentally benign nature, nonflammability, low toxicity, ready availability, and ambient critical temperature (T_c = 31.0 °C) that is suitable for biotransformations. The attraction of combining natural catalysts with a "natural" solvent has been the driving force behind a growing body of literature on the stability, activity and specificity of enzymes in $scCO_2$. The first report on biotransformations in supercritical fluids was in 1985[113–115], and the benefit of using supercritical fluids for biotransformations has been demonstrated, e.g. through improved reaction rates, *etc.*[116, 117].

Recently the alcohol dehydrogenase from *Geotrichum candidum* was found to

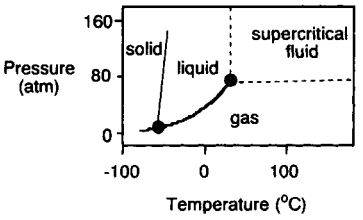


Figure 15-17. Phase diagram of carbon dioxide.

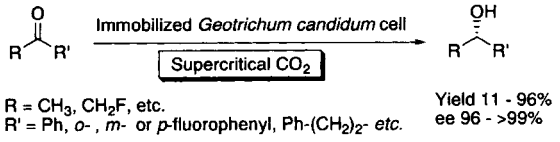


Figure 15-18. Reduction of fluoroketones by *Geotrichum candidum* IFO 5767 in supercritical CO_2[118].

catalyze the reduction of fluoroacetophenones *etc.* in $scCO_2$ at around 100 atm and 35 °C (Fig. 15-18)[118]. The enantioselectivity obtained was equivalent to the system using an organic solvent.

15.1.2.12

Modification of Reaction Conditions: Cyclodextrin

Cyclodextrin has also been used to control the enantioselectivity of bioreductions[119–121]. When added to a reaction mixture, the substrate can reside in the cyclodextrin, which decreases the effective substrate concentration around the enzyme and results in the domination of reactions involving enzymes with low K_M. The effect can be demonstrated by the reduction of ketopantoyl lactone by yeast. The enantioselectivity was improved from 73% to 93% by adding β-cyclodextrin to the reaction mixture. The improvement in enantioselectivity of the reduction in the presence of enzymes with different enantioselectivities and K_M values by decreasing the substrate concentration was confirmed by the ineffectiveness of α-cyclodextrin which is too small to include the substrate. It was also confirmed by dilution of the reaction mixture, which improved the enantioselectivity in the absence of cyclodextrin.

15.1.2.13

Modification of Reaction Conditions: Hydrophobic Polymer XAD

A decrease in the effective substrate concentration around the enzyme but not in the bulk can also be achieved using hydrophobic polymer XAD instead of using cyclodextrin or an organic solvent[122–126]. For example, the technique was used in the reduction of methyl benzyl ketone by *Zygosaccharomyes rouxii* for the synthesis of LY300164, a noncompetitive antagonist of the AMPA subtype of excitatory amino acid receptor[122]. The adsorption properties of the resin on both substrate and

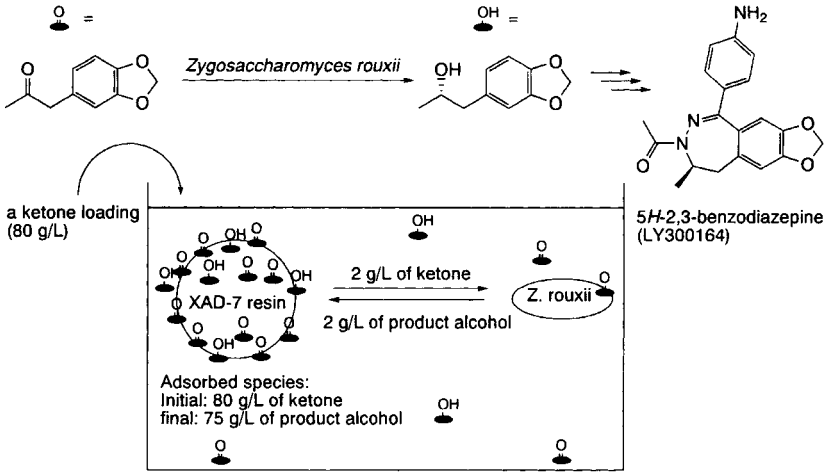


Figure 15-19. Decrease in the effective substrate concentration around the enzyme by using hydrophobic polymer XAD[122].

product allowed a ketone loading of 80 g L^{-1}, while limiting the effective solution concentration of both substrate and product to sublethal concentrations of 2 g L^{-1} (Fig. 15-19).

The hydrophobic resin has also been used for the purpose of controlling selectivity[123, 124]. Enantioselectivity, chemoselectivity and space-time yields of the yeast reduction of α,β-unsaturated carbonyl compounds were impressively enhanced. The distribution of substrates and products between the resin and the water phase showed that the improved selectivity could be attributed to the control of substrate concentration.

The powerful influence of the hydrophobic resin was also demonstrates in the *Geotrichum candidum* catalyzed reduction of simple aliphatic and aromatic ketones[126]. For example, the enantioselectivity of the reduction of 6-methylhept-5-en-2-one was improved from 27 % ee *(R)* to 98 % ee *(S)*.

15.1.2.14

Modification of Reaction Conditions: Reaction Temperature

Reaction temperature is one of the parameters that affects the enantioselectivity of a reaction[43–46]. For the oxidation of an alcohol, the values of k_{cat}/K_M were determined for the *(R)*- and *(S)*-stereodefining enantiomers; E is the ratio between them. From the transition state theory, the free energy difference at the transition state between *(R)*- and *(S)*-enantiomers can be calculated from E [Eq. (2)], and $\Delta\Delta G$ is in turn the temperature function [Eq. (3)]. The racemic temperature (T_r) can be calculated as shown in Eq. (4). With these equations, T_r for 2-butanol and 2-pentanol of the *Thermoanaerobacter ethanolicus* alcohol dehydrogenase was determined to be 26 °C and 77 °C, respectively.

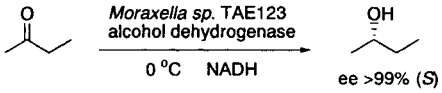


Figure 15-20. Reduction of 2-butanone by the alcohol dehydrogenase from *Moraxella sp.* TAE123 at 0 °C[17].

$$E = (k_{cat} / K_M)_R / (k_{cat} / K_M)_S \quad (1)$$

from transition state theory

$$-RT\ln(E) = \Delta\Delta G^{\ddagger} \quad (2)$$

$$\Delta\Delta G^{\ddagger} = \Delta\Delta H^{\ddagger} - T\Delta\Delta S^{\ddagger} \quad (3)$$

When

$$\Delta\Delta G^{\ddagger} = 0,\ T_r = \Delta\Delta H^{\ddagger} / \Delta\Delta S^{\ddagger} \quad (4)$$

Since the transition state for alcohol oxidation and ketone reduction must be identical, the product distribution (under kinetic control) for reduction of 2-butanone and 2-pentanone is also predictable. Thus, one would expect to isolate *(R)*-2-butanol if the temperature of the reaction was above 26 °C. On the contrary, if the temperature is less than 26 °C, *(S)*-2-butanol should result. In fact, the reduction of 2-butanone and 2-pentanone at 37 °C resulted in 28% ee *(R)- and 44% ee* (S)-alcohol, respectively, as expected[43].

The temperature range that can be used for a biocatalytic reduction is very wide because alcohol dehydrogenases from various types of microorganisms (thermophilic and psychrophilic) are available. The extremely high stability of enzymes from thermophilic microorganisms are discussed in Sect. 15.1.1.4. On the other hand, conducting reactions at temperatures as low as 0 °C is also possible using an Antarctic psychrophile[17]. For example, the reduction of 2-butanone, which is an extremely challenging substrate for enantioselective reduction, with alcohol dehydrogenase from *Moraxella sp.* TAE123, at 0 °C afforded *(S)*-2-butanol in > 99% ee (Fig. 15-20).

15.1.2.15
Modification of Reaction Conditions: Reaction Pressure

The effect of high hydrostatic pressure (400 bar) on microbial reductions of the ketones such as acetophenone, *etc.* has been examined using various strains of *Saccharomyces cerevisiae* and *Yarrowia lipolytica*. Higher enantioselectivities are generally achieved together with lower yields compared with the results obtained at atmospheric pressure as in the case of treatment of cells with high pressure homogenation[79]. Although the enantioselectivity obtained here is not as high as > 99% ee, this finding added pressure as an adjustable parameter to control the enantioselectivity of the bioreduction.

15.1.3
Improvement of Dehydrogenases for use in Reduction Reactions by Genetic Methods

15.1.3.1
Overexpression of the Alcohol Dehydrogenase

Recent developments in molecular biology have contributed to the development of useful biocatalysts. Overexpression as well as rational and random mutations of many alcohol dehydrogenases have improved the function of enzymes so that they can be useful in organic synthesis[22, 127–134]. Examples of overexpressed enzymes are introduced here, and Sect. 15.1.3.2–15.1.3.8 will describe the improvement of catalytic functions achieved by using genetic methods. Although the non-genetic chemical modifications of enzymes can also be important in order to improve a biocatalyst[135], they are not mentioned here.

Example 1: The *Thermoanaerobacter ethanolicus* 39E adhB gene encoding the secondary alcohol dehydrogenase was overexpressed in *Escherichia coli* to form more than 10% to total protein[136]. The recombinant enzyme was purified by heat treatment and precipitation with aqueous $(NH_4)_2SO_4$ and isolated in 67% yield. Enzymes with mutation(s) around the active site residues were also created to examine the catalytically important zinc binding motif in the proteins.

Example 2: The gene encoding a phenylacetaldehyde reductase with a unique and wide substrate range was cloned from the genomic DNA of the styrene-assimilating *Corynebacterium* strain ST10[137–139]. The enzyme was expressed in recombinant *E. coli* cells in sufficient quantity for practical use and purified to homogeneity by three column chromatography steps[140]. The amino acid residues assumed to be three catalytic and four structural zinc-binding ligands were characterized by site-directed mutagenesis of two zinc-binding centers within the enzyme[141].

Besides these examples, many other important enzymes for biocatalytic reductions, such as the NADPH-dependent carbonyl reductase from *Candida magnoliae*[142], the ketoreductase from *Zygosaccharomyces rouxii*[143], and the aldehyde reductase from *Sporobolomyces salmonicolor* AKU4429[144], *etc.* have also been expressed in *E. coli etc.* and shown to be active.

The availability of sufficient quantities of enzymes for crystallization studies has led to the crystal structures been obtained for several dehydrogenases. For example, two tetrameric $NADP^+$-dependent bacterial secondary alcohol dehydrogenases from the mesophilic bacterium *Clostridium beijerinckii* and the thermophilic bacterium *Thermoanaerobium brockii* have been crystallized in the apo- and the holo-enzyme forms, and their structures are available in the Protein Data Bank[145]. The crystal structure of the alcohol dehydrogenase from horse liver is also available[40–42].

15.1.3.2

Access to a Single Enzyme Within a Whole Cell: Use of Recombinant Cells

The advantages and disadvantages of using whole cell and isolated enzymes are described in Sect. 15.1.1.3. Here, genetic methods are used to build the systems with the advantages of both whole cells and isolated enzymes; the technology enables one to access essentially a single enzyme within a whole cell [127].

For example, to improve a low enantioselectivity due to the presence of plural enzymes in a cell with overlapping substrate specificities but different enantioselectivities, a recombinant cell with only the enzyme possessing the desired enantioselectivity was used (Fig. 15-21). Isolation of the enzyme, of course, improves the enantioselectivity. However, the requirement of a laborious enzyme isolation process and expensive cofactor with its associated regeneration enzyme (if necessary) have limited the practical utility of isolated enzyme processes. However, once the gene encoding the enzyme with high enantioselectivity has been overexpressed in *E. coli*, then the essentially single enzyme system can be accessed within the whole cell. Since it is a whole cell system, it can be cultivated to supply an appropriate amount without involving a laborious process for the isolation of an enzyme. The fact that there is no coenzyme requirement is also a merit for the system. Because it has only one enzyme which transforms the substrate, the problems of overmetabolism or low selectivity are also resolved. Using *E. coli* expressing Gcy1p and *E. coli* expressing Gre3p, various β-keto esters and α-alkyl-β-keto esters were reduced with excellent enantio- (up to > 98 % ee) and diastereo-selectivities (> 98 % de) [128].

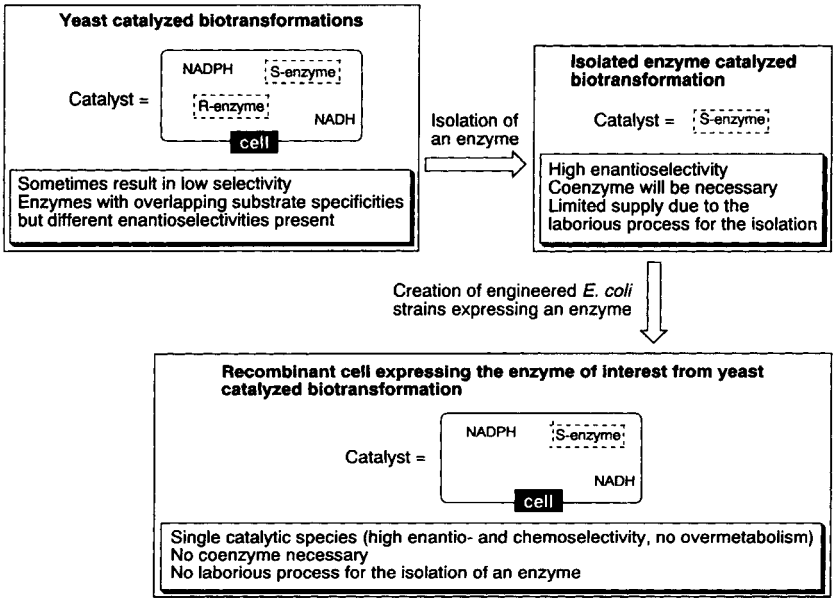


Figure 15-21. Advantages and disadvantages of whole cell, isolated enzymes and recombinant cell as biocatalysts.

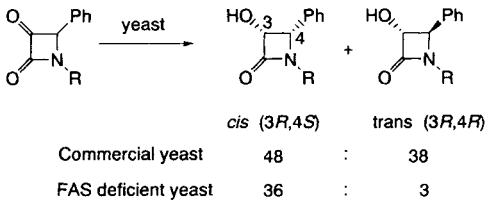


Figure 15-22. Use of FAS deficient yeast to improve the diastereoselectivity of a reduction[129].

15.1.3.3.

Use of a Cell Deficient in an Undesired Enzyme

This is a similar approach to that described above. Use of a yeast strain deficient in fatty acid synthase (FAS) suppressed formation of the undesired *trans*-diastereomer of a β-lactam as shown in Fig. 15-22[129].

15.1.3.4

Point Mutation for the Improvement of Enantioselectivity

Point mutation of enzymes has played an important role in determining those amino acid residues involved in catalytic activities. It has also been used to improve the enantioselectivity of dehydrogenases. For example, even a single point mutation of a secondary alcohol dehydrogenase from *Thermoanaerobacter ethanolicus* can change substantially the enantioselectivity for the reduction of 2-butanone and 2-pentanone as shown in Table 15-6[45].

15.1.3.5

Broadening the Substrate Specificity of Dehydrogenase by Mutations

Developments in molecular biology enable us to change the substrate specificity of enzymes; the enzymes can be engineered to be more suitable for the requisite substrate. For example, variations have been made to the structure of the NAD^+ dependent L-lactate dehydrogenase from *Bacillus stearothermophilus* (LDH)[130]. Two regions of LDH that border the active site (but are not involved in the catalytic

Table 15-6. Control of enantioselectivity by a single mutation (serine-39 to threonine) of the secondary alcohol dehydrogenase from *Thermoanaerobacter ethanolicus*[45].

Parameter	Wild type	Mutant (S39T)
k_{cat}/K_M (M^{-1} s^{-1}) for oxidation at 55 °C of:		
(*R*)-2-butanol	3.1×10^5	2.8×10^5
(*S*)-2-butanol	1.1×10^5	0.29×10^5
(*R*)-2-pentanol	0.87×10^5	3.5×10^5
(*S*)-2-pentanol	1.3×10^5	2.1×10^5
Ee of the reduction at 55 °C of:		
2-butanone	47(*R*)	81(*R*)
2-pentanone	20(*S*)	25(*R*)

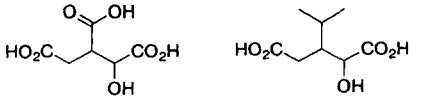
Table 15-7. Broadening the substrate specifity of L-lactate dehydrogenase from *Bacillus stearothermophilus* by rational protein engineering[130].

Enzyme	R	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M ($M^{-1}\ s^{-1}$)
Wild Type	CH_3	250	0.06	4 200 000
	$CH_2CH(CH_3)_2$	0.33	6.7	50
$^{102-105}$GlnLysPro → MetValSer	CH_3	66	0.16	410 000
	$CH_2CH(CH_3)_2$	0.67	1.9	353
$^{236-237}$AlaAla → GlyGly	CH_3	167	4	42 000
	$CH_2CH(CH_3)_2$	1.74	15.4	110
$^{102-105}$GlnLysPro → MetValSer	CH_3	32	4	8 000
/$^{236-237}$AlaAla → GlyGly	$CH_2CH(CH_3)_2$	18.5	14.3	1 300

reaction) were altered in order to accommodate substrates with hydrophobic side chains larger than that of the naturally preferred substrate, pyruvate. The mutations [102–105]GlnLysPro → MetValSer and [236–237]AlaAla → GlyGly were made to increase to tolerance for large hydrophobic substrate side chains as shown in Table 15-7. The five changes together produced a broader substrate specificity LDH, with a 55 fold improved k_{cat} for α-keto isocaproate [R = $CH_2CH(CH_3)_2$].

The substrate specificity of isocitrate dehydrogenase (IDH) has also been redesigned by genetic methods[131]. Despite the structural similarities between isocitrate (ISO) and isopropylmalate (IPM), wild type isocitrate dehydrogenase (IDH) exhibits a strong preference for its natural substrate (ISO). The substrate specificity of IDH was changed to that of isopropylmalate dehydrogenase (IPMDH) using a combination of rational and random mutagenesis. Three amino acids of IDH (S113, N115, V116) were changed and the chimeric enzyme ETV (S113E, N114T, V116V) showed

Table 15-8. Redesigning the substrate specificity of isocitrate dehydrogenase[131].



isocitrate (ISO) isopropylmalate (IPM)

Enzyme	IDH position 113	115	116	k_{cat}/K_M IPM ($M^{-1}\ s^{-1}$)	k_{cat}/K_M ISO ($M^{-1}\ s^{-1}$)	k_{cat}/K_M IPM / k_{cat}/K_M ISO
Wild Type IPMDH	E	L	L	1.4×10	0	–
Wild Type IDH	S	N	V	1.7×10^{-6}	1.6×10	1.0×10^{-7}
EVG	E	V	G	1.1×10^{-5}	1.1×10^{-5}	1.0
ENA	E	N	A	1.5×10^{-5}	5.9×10^{-6}	2.5
ETV	E	T	V	1.8×10^{-4}	3.9×10^{-5}	4.6

Table 15-9. Elimination of the cofactor requirement by "blind" directed evolution[132].

Bacillus stearothermophillus lactate dehydrogenase	Cofactor (Fructose 1,6-bisphosphate)	$K_M^{pyruvate}$ (mM)
Wild	+	0.05
Wild	–	5
Mutated (R118C, 203L, N307S)	+	0.05
Mutated (R118C, 203L, N307S)	–	0.07

a preferred substrate specificity for IPM over ISO; $[k_{cat}/K_M IPM]$ / $[k_{cat}/K_M ISO]$ of ETV was 4.6 while that of wild type IDH was 1.0×10^{-7}.

15.1.3.6
Production of an Activated Form of an Enzyme by Directed Evolution

One of the drawbacks of using alcohol dehydrogenases as catalysts for organic synthesis (comparing them with hydrolytic enzymes) is the cofactor requirement[132]. For example, *Bacillus stearothermophillus* lactate dehydrogenase is activated in the presence of fructose 1,6-bisphosphate[132]. The activator is expensive and representative of the sort of cofactor complications that are undesirable in industrial processes. Three rounds of random mutagenesis and screening produced a mutant which is almost fully activated in the absence of fructose 1,6-bisphosphate as shown in Table 15-9.

15.1.3.7
Change in the Coenzyme Specificity by Genetic Methods: NADP(H) Specific Formate Dehydrogenase

Formate/formate dehydrogenase is one of the most useful coenzyme regeneration systems as has been described in the Sect. 15.1.1.2. However, the known wild type formate dehydrogenases only accept NAD^+; $NADP^+$ is not the substrate. Multipoint site-directed mutagenesis was used to create a formate dehydrogenase which was able to accept $NADP^+$. This mutant enzyme was then coupled to the reduction using the alcohol dehydrogenase from *Lactobacillus sp* as shown in Fig. 15-23[22]. The activity of the NADP(H)-specific mutant (with $NADP^+$ as substrate) is about 60% of the activity of wild type formate dehydrogenase (with NAD^+ as substrate).

15.1.3.8
Use of a Mutant Dehydrogenase for the Synthesis of 4-Amino-2-Hydroxy Acids

The usefulness of a mutant dehydrogenase was demonstrated in a practical synthesis of 4-amino-2-hydroxy acids, which themselves are valuable as γ-turn mimics for investigations into the secondary structure of peptides[146]. Chemoenzymatic synthesis of these compounds were achieved by lipase catalyzed hydrolysis of a α-keto esters to the corresponding α-keto acids followed by reduction employing a lactate dehydrogenase in one pot. Wild type lactate dehydrogenase from either *Bacillus*

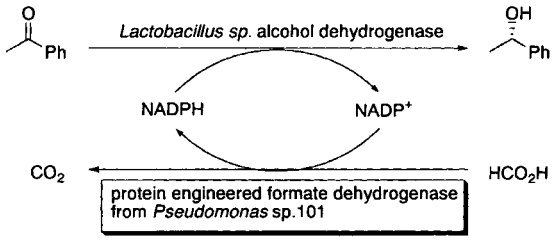
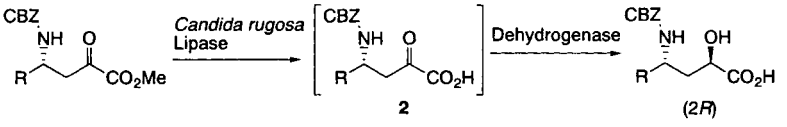


Figure 15-23. Recycling of NADPH with protein engineered formate dehydrogenase[22].

Table 15-10. The use of a mutant dehydrogenase for the synthesis of 4-amino-2-hydroxy acids[146].



Dehydrogenase	R	Reaction Time	Yield (%)
Wild type	*a:* CH_3	4 days	67
Staphylococcus epidermidis	*b:* $CH(CH_3)_2$	no reaction	–
lactate dehydrogenase	*c:* $CH_2CH(CH_3)_2$	no reaction	–
	d: CH_2Ph	no reaction	–
H205Q mutant of	*a:* CH_3	4 h	85
Lactobacillus delbrueckii bulgaricus	*b:* $CH(CH_3)_2$	5 h	90
D-hydroxyisocaproate dehydrogenase	*c:* $CH_2CH(CH_3)_2$	4 h	78
	d: CH_2Ph	5 h	85

stearothermophilus (BS-LDH) or *Staphylococcus epidermidis* (SE-LDH) could be used specifically to reduce the ketone of the alanine derived α-keto acid, **2a**, giving the *(S)*- and *(R)*-2-hydroxy acids, respectively, in good yields.

However, more bulky α-keto acids **2b–2d** were not substrates for these enzymes. In contrast, the genetically engineered H205Q mutant of *Lactobacillus delbrueckii bulgaricus* D-hydroxyisocaproate dehydrogenase proved to be an ideal catalyst for the reduction of all the α-keto acids **2a–2d**, giving excellent yields of the CBZ-protected (2*R*, 4*S*)-4-amino-2-hydroxy acid as a single diastereomer (Table 15-10). This genetically engineered oxidoreductase has great potential value in synthesis, not only due to its broad substrate specificity but also due to the high catalytic activity. For example, reduction of 1 mmol of **2a** took just 4 h with the H205Q mutant, whereas with SE-LDH the reaction required 4 days.

15.1.3.9
Catalytic Antibody

Nakayama and Schultz have developed antibodies to carry out the catalytic enantioselective reduction of an α-keto amide using $NaBH_3CN$ as the reductant[147]. Monoclonal antibodies raised to phosphonate **3** were prepared (Fig. 15-24), and one antibody showed activity for the enantioselective reduction of a chiral keto amide **4**.

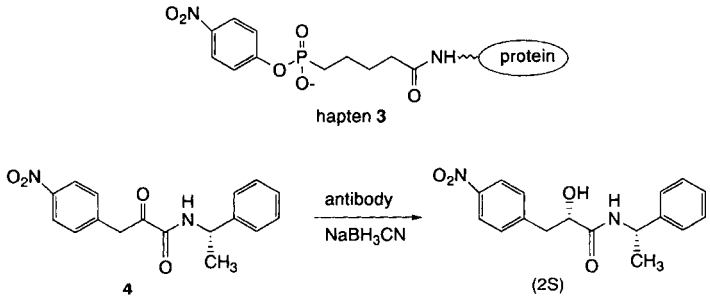


Figure 15-24. Reduction of a ketone by a catalytic antibody[147].

Reduction with the antibody gave the 2*S* product with a diastereomeric excess greater than 99% (opposite to the stereoselectivity of the uncatalyzed reaction which afforded the 2*R* product).

15.1.4
Reduction Systems with Wide Substrate Specificity

15.1.4.1
Bakers' Yeast

Many methods for asymmetric reduction have been developed and some of these are used for the synthesis of optically active alcohols on a preparative scale. Bakers' yeast is one of the most widely used microorganisms due to its commercial availability and its wide substrate specificity, which enables the non-expert in biochemistry to use the biocatalyst as a reagent for organic synthesis. Detailed reactions will not be described in this text since there are many reviews and original reports on this subject[1, 37–39, 148–162]. However, one of the most important and useful reactions using yeast, the reduction of a hydroxymethyl ketone, is featured here due to the excellent enantioselectivity obtained even on a large scale (Fig. 15-25).[163–166]. For example, 1-hydroxy-2-heptanone (50 g) was reduced to the corresponding *(R)*-diol in an optically pure form in 56% yield [Fig. 15-25 (b)][164]. Another example [Fig. 15-25 (c)] is the reduction of a sulphenyl hydroxyketone with yeast in the synthesis of a natural product[166]. Products isolated from the mandibular glands of the oriental hornet were synthesized using yeast reduction of an S-substituted hydroxyketone.

15.1.4.2
Rodococcus erythropolis

A carbonyl reductase isolated form *Rhodococcus erythropolis* accepts a broad range of substrates, including a variety of compounds useful for synthetic chemistry, as shown in Table 15–11[25]. Reduction of all the carbonyl compounds tested yielded *(S)*-configured hydroxyl compounds with high enantioselectivities.

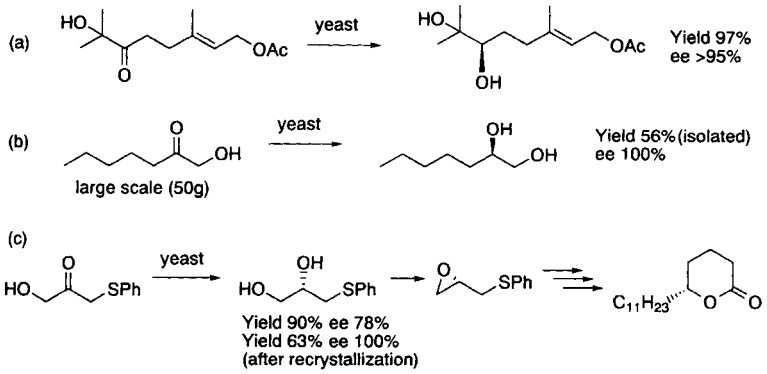


Figure 15-25. Reduction of hydroxyketones by bakers' yeast[163, 164, 166].

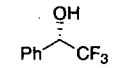
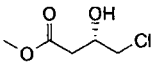
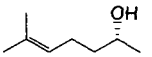
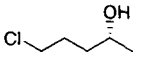
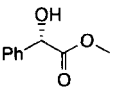
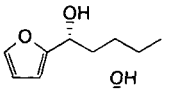
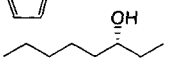
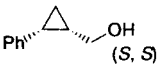
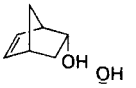
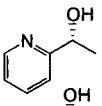
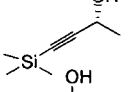
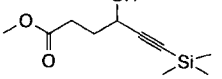
Table 15-11. Kinetic constants of the *R. erythropolis* carbonyl reductase[25].

Substrate	V_{max} (U mg^{-})	K_M (m*M*)	Substrate	V_{max} (U mg^{-1})	K_M (m*M*)
	3.5	330		0.46	18
	3.5	260		1.4	7.3
	4.8	59		2.6	16
	7.7	3.8		5.5	3.1
	10.4	0.59		4.2	9.9
	10.3	0.42		7.6	8.3
	10.8	0.34		10.6	0.039
	11.1	0.54		1.7	3.8

15.1.4.3
Pseudomonas sp. Strain PED and *Lactobacillus kefir*

The substrate specificities of the alcohol dehydrogenases from *Pseudomonas sp.* strain PED and *Lactobacillus kefir* have been investigated. It was reported that they reduce wide varieties of ketones[6, 7]. Both reactions use 2-propanol for the regeneration of coenzyme and produce *(R)*-alcohols as depicted in Table 15-12. However, they require different coenzymes. The alcohol dehydrogenase from the *Pseudomonas sp.* uses NADH and transfers to pro-*R* hydride of NADH to the *si*-face of carbonyl compounds as shown in Sect. 15.1.1.1. The mechanism is ordered bi–bi with the coenzyme binding first and released last. On the other hand, the enzyme from

Table 15-12. Enantioselectivities of the alcohol dehydrogenases from *Pseudomonas sp.* strain PED and *Lactobacillus kefir*[6, 7].

Product	ee (%)		Product	ee (%)	
	***Pseudomonas sp.* strain PED**	***Lactobacillus kefir***		***Pseudomonas sp.* strain PED**	***Lactobacillus kefir***
	92	> 99		98	–
	94	–		97	> 99
	86	–		93	> 97
	98	–		45	–
				27	–
	65	–		–	> 97
	92	–		–	> 97
	–	> 97		–	94
	–	95		–	97

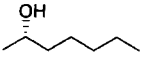
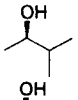
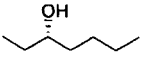
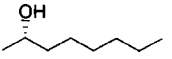
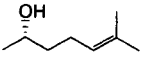
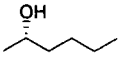
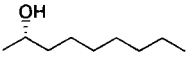
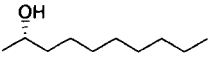
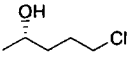
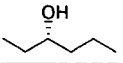
Lactobacillus kefir uses NADPH and transfers the *pro-R* hydride from the cofactor to the *si*-face of carbonyl compounds.

15.1.4.4
Thermoanaerobium brockii

The alcohol dehydrogenase from *Thermoanaerobium brockii* is very suitable for the reduction of aliphatic ketones[18, 19]. Even very simple aliphatic ketones can be reduced enantioselectively. An interesting substrate size-induced reversal of enantioselectivity was observed. The smaller substrates (methyl ethyl, methyl isopropyl or methyl cyclopropyl ketones) were reduced to the *(R)*-alcohols, whereas higher ketones produced the *(S)*-enantiomers.

This example and the next one (Sect. 15.1.4.5) using *G. candidum* show that the biocatalytic reduction system is very beneficial for the reduction of aliphatic ketones over a non-enzymatic system where no report on highly enantioselective (> 99% ee) reduction of unfunctionalized dialkyl ketones can be found, to the best of our knowledge.

Table 15-13. Asymmetric reduction of aliphatic ketones with the alcohol dehydrogenase from *Thermoanaerobium brockii*[18].

Product	Relative rate	ee (%)	Config.	Product	Relative rate	ee (%)	Config.
	12.0	48	*R*		0.9	99	*S*
	3.0	86	*R*		0.2	95	*S*
	0.8	44	*R*		0.6	97	*S*
	3.3	79	*S*		0.3	99	*S*
	1.0	96	*S*		0.3	98	*S*
	0.3	95	*S*		0.1	99	*S*
	0.1	81	2 *S*, 3 *R*		1.5	98	*S*
	0.9	97	*S*				

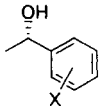
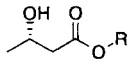
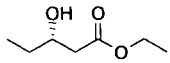
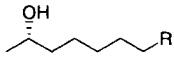
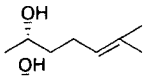
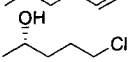
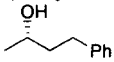
15.1.4.5
Geotrichum candidum

Reductions using an acetone powder of *G. candidum* (APG4), NAD^+ and 2-propanol exhibit one of the widest substrate specificities together with very high enantioselectivities (Table 15-14)[20, 21]. Various ketones such as acetophenone derivatives can be reduced with APG4 with excellent enantioselectivities (> 99% ee). The nature and electronegativity of substituents on the phenyl ring did not affect the enantioselectivity although the yield was slightly lower for *para* derivatives than for the corresponding *ortho* and *meta* derivatives.

Reduction by APG4 of several aromatic ketones having different length alkyl chains demonstrated the scope and limitations of the substrate specificity. The phenyl moiety of acetophenone can be replaced by a benzyl or even by a 2-phenylethyl group with slightly better results in terms of chemical yield without any decrease in enantioselectivity. However, when the methyl moiety of actophenone was replaced by an ethyl, isopropyl or methoxymethyl group, the yield decreased dramatically, although the enantioselectivity remained high (> 99% ee). When the alkyl chain was elongated to a propyl or enlarged to a *t*-butyl group, the reaction was observed scarcely to proceed.

The versatility of the APG4 reduction system is further exemplified by the use of β-keto esters as substrates. 3-Oxobutyrates involving methyl, ethyl, *t*-butyl, or neopentyl esters are reduced to the *(S)*-hydroxyesters with > 99% ee and in quantitative yield. Moreover, simple aliphatic ketones from 2-octanone to 2-undecanone, as well

Table 15-14. Reduction of various ketones by the acetone powder of *G. candidum*, NAD^+ and 2-propanol[20, 21].

Product	Yield (%)	ee (%)	Product	Yield (%)	ee (%)
	X = H 89	> 99 (*S*)		R = Et 41	> 99 (*S*)
	o-F > 99	> 99 (*S*)		Pr 0	–
	m-F 95	> 99 (*S*)		*i*-Pr 12	99 (*S*)
	p-F 74	> 99 (*S*)		*t*-Bu 1	–
	o-Cl > 99	> 99 (*S*)		CH_2OMe 8	> 99 (*R*)
	m-Cl 95	99 (*S*)		CH_2Cl 80	98 (*R*)
	p-Cl 62	> 99 (*S*)			
	o-Br 97	> 99 (*S*)		R = Me > 99	> 99 (*S*)
	m-Br 92	> 99 (*S*)		Et > 99	> 99 (*S*)
	p-Br 95	> 99 (*S*)		*t*-Bu > 99	> 99 (*S*)
	o-Me 96	> 99 (*S*)		neo-Pentyl > 99	> 99 (*S*)
	m-Me 86	> 99 (*S*)		72	> 99 (*S*)
	p-Me 78	> 99 (*S*)			
	o-MeO 84	> 99 (*S*)			
	m-MeO 90	> 99 (*S*)		R = me 87	> 99 (*S*)
	p-MeO 29	> 99 (*S*)		Et 87	> 99 (*S*)
	o-CF_3 6	97 (*S*)		Pr 85	> 99 (*S*)
	m-CF_3 96	> 99 (*S*)		Bu 60	> 99 (*S*)
	p-CF_3 73	> 99 (*S*)			
	1',2',3',4',5'-F_5 62	> 99 (*S*)		90	99 (*S*)
	96	> 99 (*S*)		92	99 (*S*)
	93	> 99 (*S*)			

as 6-methyl-5-heptene-2-one and 5-chloro-2-pentanone are also reduced by the APG4 system to the corresponding *(S)*-2 alkanols giving high yields with 99% ee.

In summary, a detailed investigation of substrate specificity for the acetone powder of a *G. candidum* system reveals that as long as there is a methyl group at the α-position of the carbonyl group, high yield and enantioselectivity can be obtained regardless of the substituent on the other side of the ketone moiety.

Apart from acetone-dried *G. candidum* IFO 4597, intact whole cells of various strains of *G. candidum* have been found to be useful for asymmetric reductions[75–78, 101, 126, 167–171]. For example, methyl 2-acetylbenzoate was reduced by *G. candidum* ATCC 34614, IFO 5767 or IFO 4597 as well as by other microorganisms such as *Mucor javanicus, Mucor heimalis, Endomyces magnusii, Endomyces reessii* and bakers' yeast to afford phthalide derivatives (Fig. 15-26) which have various pharmacological profiles such as relaxant, antiproliferative or antiplatelet effects, *etc.*[171].

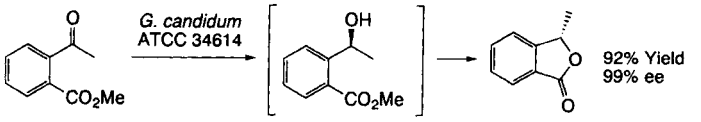


Figure 15-26. Asymmetric reduction by *G. candidum* ATCC 34614 for the synthesis of a bioactive phthalide derivative[171].

15.1.5
Reduction of Various Ketones

15.1.5.1
Reduction of Fluoroketones

The biocatalytic reduction of fluoroketones is useful in order to gain an insight into the enzyme recognition of fluorinated groups, and is also very important due to the high synthetic values of the products, optically active fluorinated alcohols[160, 172–185]. Sometimes the monofluorinated substrate can be a straightforward mimic of the unsubstituted counterpart, but with difluorinated and trifluorinated substrates, different recognition patterns compared with unfluorinated or monofluorinated substrates and with each other are often observed. For example, the enantioselectivity of yeast reduction is definitely affected by the fluorination pattern on the substrate[172]. One of the most prominent effects of the fluorination of a substrate is seen in the reduction of acetophenone derivatives by the acetone powder of *Geotrichum candidum* (APG4) as shown in Fig. 15-27[173, 174]. Reduction of methyl ketones afforded *(S)*-alcohols in excellent ee, whereas the reduction of trifluoromethyl ketones gave the corresponding alcohols of the opposite configuration, also in excellent ee. Monofluoroacetophenone and difluoroacetophenone were also reduced under the same conditions. The reduction proceeded quantitatively for both substrates. As expected, the stereoselectivity shifted from the acetophenone type to the trifluoroacetophenone type according to the number of fluorine substituents at the α-position as shown in Fig. 15-28.

The replacement of the methyl moiety with a trifluoromethyl group alters the bulkiness and electronic properties: the effect on the enantioselectivity has been examined. No inversion in stereochemistry was observed for the reduction of hindered ketones such as isopropyl ketone, while the stereoselectivity was inverted for the reduction of ketones with electron-withdrawing atoms such as chlorine. The mechanism for the inversion in stereochemistry was investigated in further studies. Several enzymes with different enantioselectivities were isolated; one of them

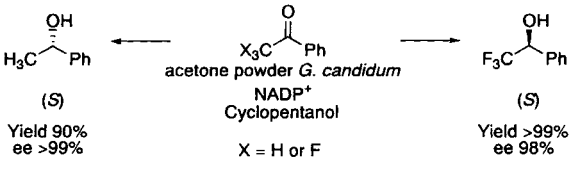


Figure 15-27. Reduction of acetophenone and trifluoroacetophenone by an acetone powder of *Geotrichum candidum*, $NADP^+$ and cyclopentanol[173, 174].

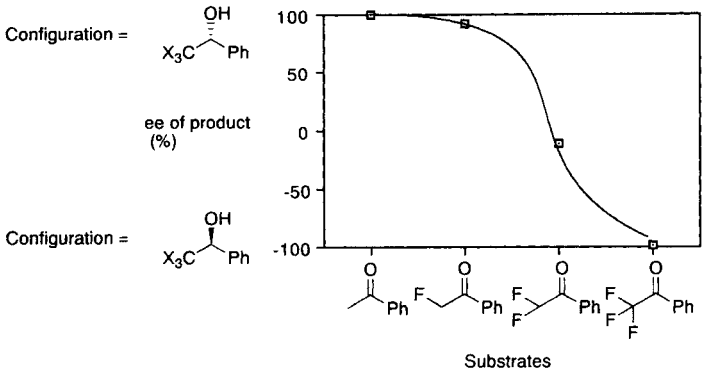


Figure 15-28. Effect of introducing a fluorine atom or atoms at the α-position of acetophenone on the stereoselectivity in the reduction by *G. candidum* acetone powder[174].

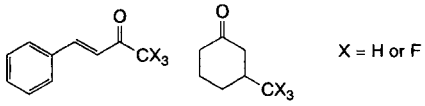


Figure 15-29. Substrates used for the examination of the stereodirecting effects of trifluoromethyl and methyl groups[175].

catalyzed the reduction of methyl ketones, and another, with the opposite enantioselectivity, catalyzed the reduction of trifluoromethyl ketones.

The differing abilities of trifluoromethyl and methyl groups to direct enantioselection in the reduction of carbonyl substrates has also been analyzed using various other microorganisms including different strains of *G. candidum, Hansenula anomala, Saccharomyces cervisiae, Streptomyces, etc.*[175]. The reduction of the cyclic ketone and enones shown in Fig. 15-29 was investigated. The differences in the electronic and steric properties of the trifluoromethyl and methyl residues resulted in different chemo- and enantioselectivities in the reduction of the phenylbutenones, while the cyclohexanones showed similar enantioselectivities.

Many synthetically valuable reactions involving reductions of fluoroketones have been reported as shown in Fig. 15-30[176–178]. Various monofluoroketones are reduced with yeast; some of them proceeded with high diastereoselectivity.

Chiral trifluoromethyl benzyl alcohols are useful synthons for ferroelectric liquid crystals. Therefore, Fujisawa *et al.* investigated the asymmetric reduction of the corresponding ketones using bakers' yeast[179, 180]. The enantioselectivity of the bakers' yeast reduction of trifluoroacetylbenzene derivatives was improved by the introduction of some functional groups at the *para*-position to give the corresponding *(R)*-trifluoromethyl substituted benzylic alcohols in high chemical and optical yields as shown in Fig. 15-31. The "enantio-controlling" functional group at the *para*-position was then used in further transformations.

Yeast and *G. candidum* acetone powder (APG4) are complementary to each other in the reduction of various trifluoromethyl biphenyl ketones. Yeast reduction affords the *(R)*-alcohol, whereas *G. candidum* reduction affords the *(S)*-alcohol (Fig. 15-32)[181].

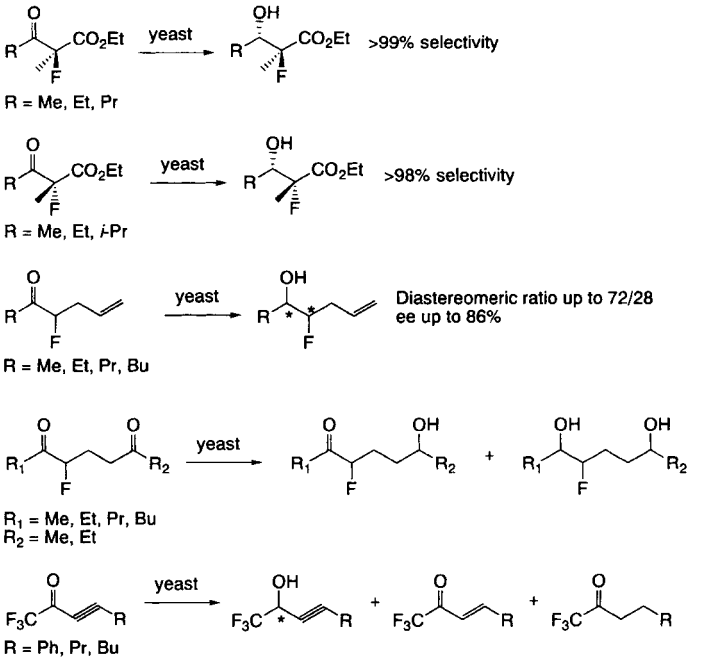


Figure 15-30. Reduction of fluorinated ketones by yeast[176–178].

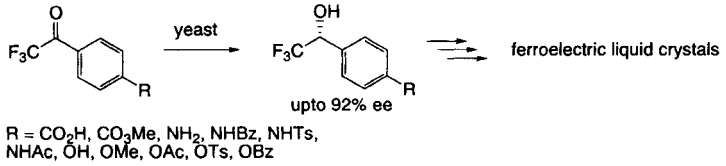


Figure 15-31. Asymmetric reduction of trifluoroacetylbenzene derivatives by bakers' yeast[179, 180].

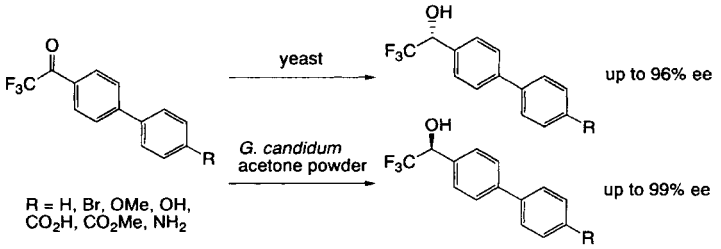
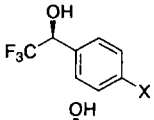
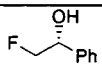
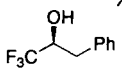
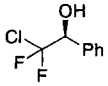


Figure 15-32. Reduction of trifluoromethyl biphenyl ketones: bakers' yeast vs *G. candidum* acetone powder[181].

Moreover, various optically pure fluorinated alcohols are produced by employing *G. candidum* reductions as shown in Table 15-15[174]. Monofluoroacetophenone and difluoroacetophenone are reduced to *(R)*-alcohols by the acetone powder, NAD^+ and

Table 15-15. Synthesis of chiral fluorinated alcohols by the reduction with acetone powder and isolated enzymes of *Geotrichum candidum* IFO 4597[174].

Product		Yield (%)	ee (%)	Product	Yield (%)	ee (%)
	X = H X = Cl X = Br	84 81 80	98 (*S*) > 99 (*S*) > 99 (*S*)		93	> 99 (*R*)
					91	> 99 (*S*)[a]
		74	98 (*S*)		99	63 (*R*)
		82	94 (*S*)		95	> 99 (*S*)[a]

a The isolated enzyme was used for the reduction.

2-propanol, and to (*S*)-alcohols by a constituent enzyme previously separated by anion-exchange chromatography and using glucose-6-phosphate/glucose-6-phosphate dehydrogenase as the cofactor recycling system. Both enantiomers of monofluorophenylethanol can be obtained with excellent ee using only one microorganism.

15.1.5.2
Reduction of Fluoroketones Containing Sulfur Functionalities

As the demand for optically active fluorinated compounds increases, the importance of the development of asymmetric synthetic methods for fluorinated building blocks grows. On the other hand, sulfur functionalities such as phenylthio and dithianyl groups have been used as useful reactive units for a variety of chemical transformations. Therefore, various trifluoromethyl ketones containing a sulfur functionality have been reduced with various microorganisms[182–185].

For example, several microorganisms have been employed for the reduction of α,α,α,-trifluoromethyl α'-sulphenyl ketones (Fig. 15-33). Some of them produce the corresponding alcohols in high diastereo- and enantioselectivities; the high conver-

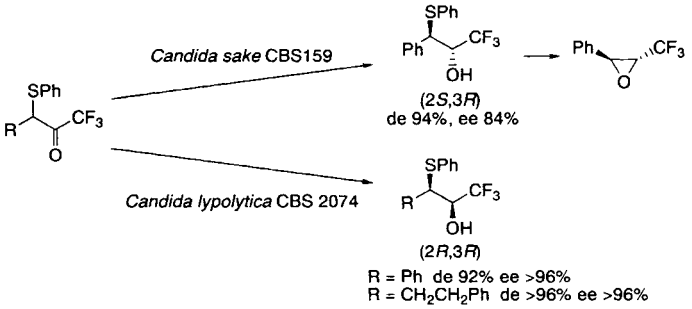
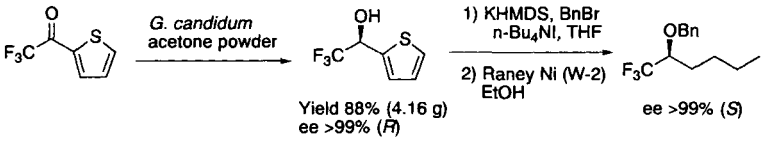
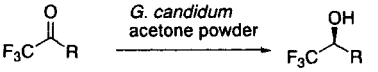


Figure 15-33. Reduction of sulphenyl ketones followed by epoxide formation[182].





R	Yield (%)	ee (%)
SPh	90	98 (*R*)
$CH_2CH_2CH_2SPh$	78	>99 (*S*)
$CH_2SC_9H_{17}$	35	96 (*R*)
3-Thienyl	87	>99
1,3-Dithian-2-yl	42	>99

Figure 15-34. Asymmetric reduction of trifluoromethyl ketones containing a sulfur functionality by the acetone powder of *G. candidum*[183].

sion into a single enantiomer is secured by the racemization of starting ketones under the biotransformation conditions. Transformation of the resulting sulphenyl trifluoromethyl alcohols into trifluoromethyl epoxides was also achieved[182].

The acetone powder of *G. candidum* (APG4) has also been used for the reduction of sulfur containing trifluoromethyl ketones (Fig. 15-34)[183]. This reaction can be scaled up easily without the loss of enantioselectivity. For example, the reduction of trifluoro(2-thienyl)ethanone on the gram scale proceeded quantitatively and yielded the optically pure *(R)*-alcohol in 88% yield after purification (4.16 g, ee > 99%). The thienyl alcohol can be further transformed into a fluorinated aliphatic alcohol without racemization.

15.1.5.3
Reduction of Chloroketones

The reduction of chloroketones has been widely investigated since it can produce versatile chiral intermediates. For example, reduction of an α-chloroketone results in the formation of a chlorohydrin, which can easily be transformed into an epoxide on treatment with a base. On recently published example involves the reduction of 3,4-dichlorophenacylchloride by *Rhodotorula mucillaginosa* CBS 2378 or *Geotrichum candidum* CBS233.76 to give the *(R)*- or *(S)*-chlorohydrin with > 99% ee and > 98% ee, respectively, as shown in Fig. 15-35[186]. The *(S)*-enantiomer was transformed into the corresponding epoxide and then into a dichlorophenylbutanolide, an intermediate in the synthesis of (+)-*cis*-1*S*,4*S*-sertraline, which is an antidepressant drug of the selective serotonin reuptake inhibitor (SSRI) type.

There are also many other examples of the reduction of α-halomethyl ketones as shown in Table 15-16[187–189]. Various microorganisms are able to reduce fluoro-, chloro- and bromoketones[161, 190–192]. However, reduction of iodoacetophenone usually results in a poor yield, producing, mainly, acetophenone or phenylethanol.

Another example of the reduction of α-chloroketone involves dynamic kinetic resolution. The reduction of an α-chloroketo ester by *M. racemosus* and *R. glutinis* resulted in optically active *syn*- and *anti*-chlorohydrin, respectively, as shown in

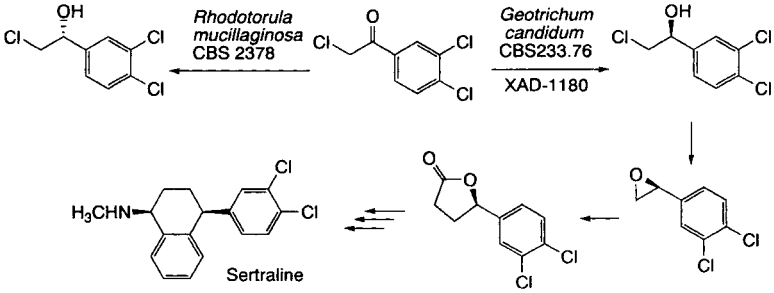
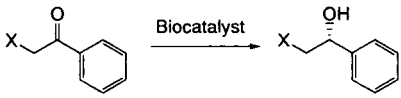


Figure 15-35. Reduction of a chloroketone followed by epoxidation for the synthesis of sertraline[186].

Table 15-16. Reduction of α-halogenated acetophenones.



Catalyst	X	Yield[a] (%)	ee (%)	Reference
Cryptococcus macerans	Cl	80	100	187
	Br	95	93	187
Bakers' yeast	F	67	97	188
	Cl	37	90	188
	Br	9	97	188
	F	55	35	189
	Cl	6 (40)	68	189
	Br	0 (15)	–	189
Geotrichum candidum sp. 38	F	65	75	189
	Cl	86	87.4	189
	Br	15 (25)	94	189

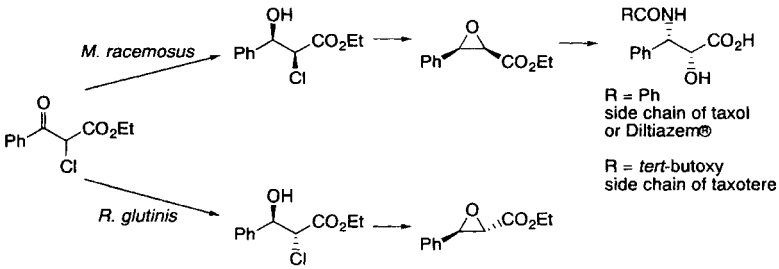
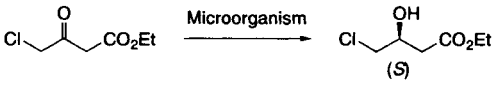


Figure 15-36. Enantio- and diastereo-selective reduction of a chloroketone[193, 194].

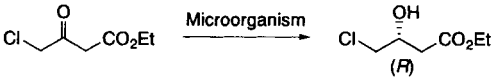
Fig. 15-36[193]. The *syn*-isomer was transformed into the corresponding epoxide, followed by conversion into the side chain of taxol and taxotere[194].

One of the most studies α-chloroketones is ethyl 4-chloro-3-oxobutanoate. *(R)*- and *(S)*-enantiomers of the corresponding alcohol were produced by various micro-

Table 15-17. Comparison of various microorganisms for the reduction of ethyl 4-chloro-3-oxobutanoate.



Microorganism	Yield (%)	ee (%)	Reference
Geotrichum candidum	98	96	170
Bakers' Yeast	100	90	90
Bakers' Yeast		55	61
Lactobacillus kefir	100	100	195
Candida magnoliae (recombinant and overexpressed in *Escherichia coli*)	88	100	142



Microorganism	Yield (%)	ee (%)	Reference
Dancus carota	42	52	196
Sporobolomyces salmonicolor	95	86	197
Lactobacillus fermentum	70	98	195
Saccharomyces cerevisiae (FAS (β-keto reductase) negative)	55	16	63

organisms as shown in Table 15-17. The *(R)*-enantiomer is a promising chiral building block for the synthesis of L-carnitine, an essential factor for the β-oxidation of fatty acids in mitochondria.

As shown in Fig. 15-37, a chiral intermediate for a human immunodeficiency virus protease inhibitor (HIVPI) was also synthesized by the reduction of an α-chloroketone with a *Streptomyces* strain [198].

Another example of the reduction of chloroketone is the reduction of 5-chloro-2-pentanone by TBADH as shown in Fig. 15-38 [19]. Using this biotransformation in the synthetic pathway, a naturally occurring heterocycle isolated from the glandular secretion of the civet cat *(Viverra civetta)*, was prepared.

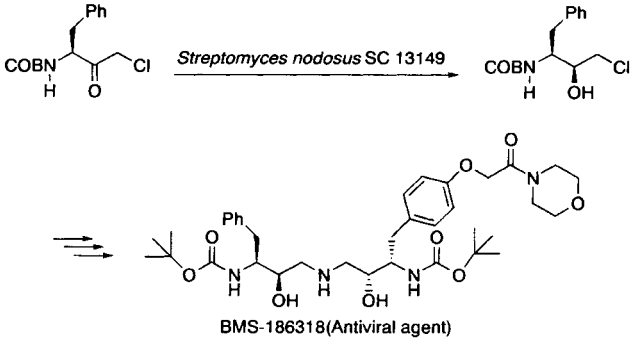


Figure 15-37. Synthesis of a chiral intermediate for an HIV-PI [198].

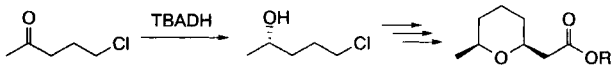


Figure 15-38. Reduction of 5-chloro-2-pentanone by TBADH for natural product synthesis[19].

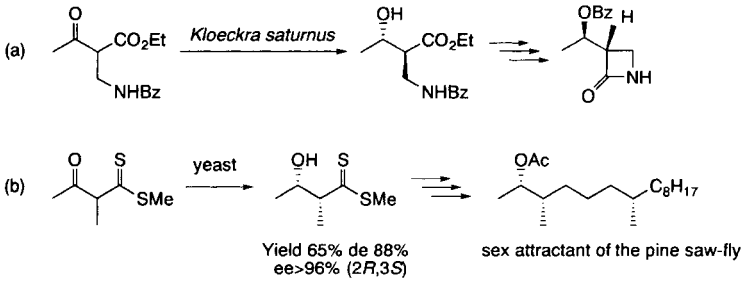


Figure 15-39. Reduction of ketones containing sulfur or nitrogen functionality[199, 219].

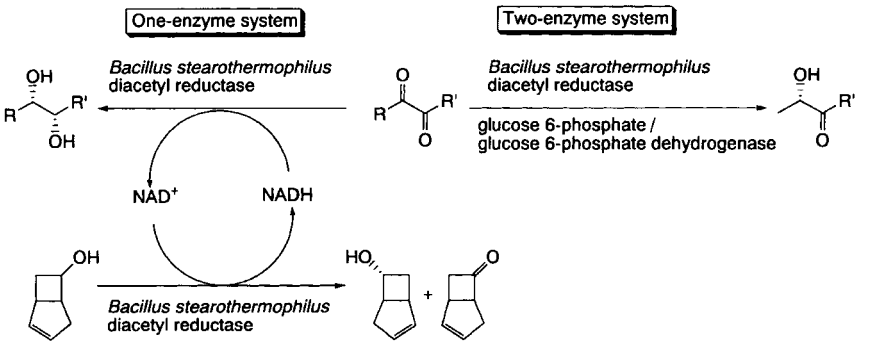
15.1.5.4
Reduction of Ketones Containing Nitrogen, Oxygen, Phosphorus and Sulfur Functionalities

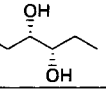
Ketones with useful heteroatomic functional groups containing nitrogen[199–212], oxygen[163, 213–217], phosphorus[218] and sulfur[154, 184, 219–227] have been reduced by biocatalysts. For example, an intermediate in the synthesis of β-lactam antibiotics was obtained by microbial reduction of a β-keto ester as shown in Fig. 5-39(a)[199], while yeast reduction of a β-keto dithioester afforded an easily separable mixture of β-hydroxy-dithioesters, the major component of which was converted enantioselectively into a sex attractant of the pine saw-fly as shown in Fig. 15-39(b)[219].

15.1.5.5
Reduction of Diketones

Regio- and enantioselective reduction of diketones can be achieved readily by using a biocatalyst[228–242]. As a result, optically active hydroxyketones and diols have been synthesized successfully.

For the reduction of α-diketones, the selectivity between the reduction to diol and to hydroxyketone can be controlled using a diacetyl reductase from *Bacillus stearothermophilus* (Fig. 15-40)[233]. When a one-enzyme system was used for the coenzyme recycling using endo-bicyclo[3.2.0]hept-2-en-6-ol (**5**), both carbonyl groups were reduced selectively to produce a diol. On the other hand, α-hydroxyketones were obtained using a two-enzyme system glucose 6-phosphate/glucose 6-phosphate dehydrogenase for coenzyme recycling. The synthetic potential of both systems has been illustrated by the synthesis of the male sex pheromone of the grape borer *Xylotrechus pyrrhoderus*, identified as a two-component mixture of the reduction products, **6** and **7**.



Product		Yield (%)	ee (%)
	R = Me	40	>98 (*S,S*)
	Pr	92	>98 (*S,S*)
	Ph	80	>98 (*S,S*)
	Pentyl (6)	82	>98 (*S,S*)
		80	95 (*S,S*)

Product		Yield (%)	ee (%)
	R = Pr	96	>98 (*S*)
	Ph	92	>98 (*S*)
	Pentyl (7)	65	>98 (*S*)

Figure 15-40. Reduction of α-diketones by diacetyl reductase from *Bacillus stearothermophilus*[233].

Regio- and enantioselective reduction of β-diketones may be carried out using biocatalysts. For example, a diketo ester **8** was reduced by the alcohol dehydrogenase from *Lactobacillus brevis*, to provide the corresponding hydroxyketo ester with 99.4% ee in 78% yield; this was used as an intermediate for the synthesis of dimeric metabolite vioxanthin of *Penicillium citreo-viride* in order to develop an assay system to monitor phenol oxidative coupling in lignan formation [Fig. 15-41(a)][228]. Yeast reduction also proceeds regio- and enantioselectively with aliphatic diketones producing hydroxyketones with perfect selectivities as shown in Fig. 15-41(b)[232]. The yeast reduction also proceeds satisfactorily with 2,2-disubstituted cycloalkanediones, producing hydroxyketones with excellent enantio- and diastereoselectivities as shown in Fig. 15-41(c)[231].

15.1.5.6
Reduction of Diaryl Ketones

Bulky ketones such as diaryl ketones can be also reduced by biocatalysts. For example, a rice plant growth regulator, *(S)*-N-isonicotinoyl-2-amino-5-chlorobenzhydrol, was prepared by microbial reduction of 2-amino-5-chlorobenzophenone with *Rhodosporidium toruloides* followed by isonicotinoylation as shown in Fig. 15-42(a)[243]. A phosphodiesterase 4 inhibitor was also prepared by microbial reduction of a diaryl ketone **9** with *Rhodotorula pilimanae*, which was found by the screening of 310 microbial strains [Fig. 15-42(b)][244].

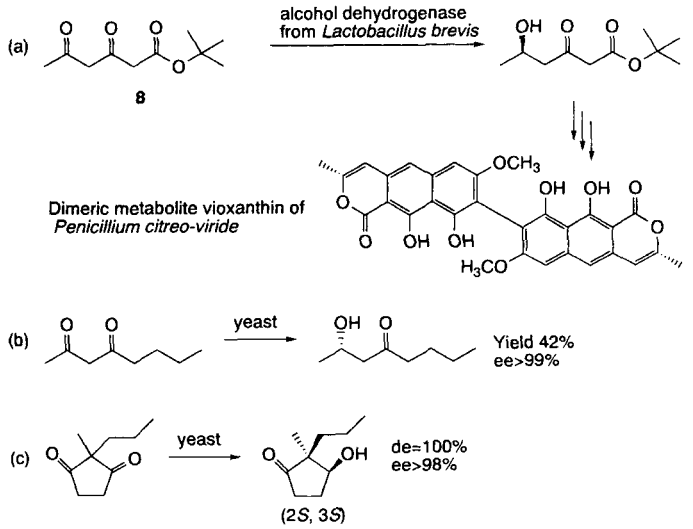


Figure 15-41. Regio- and enantioselective reduction of diketones[228, 231, 232].

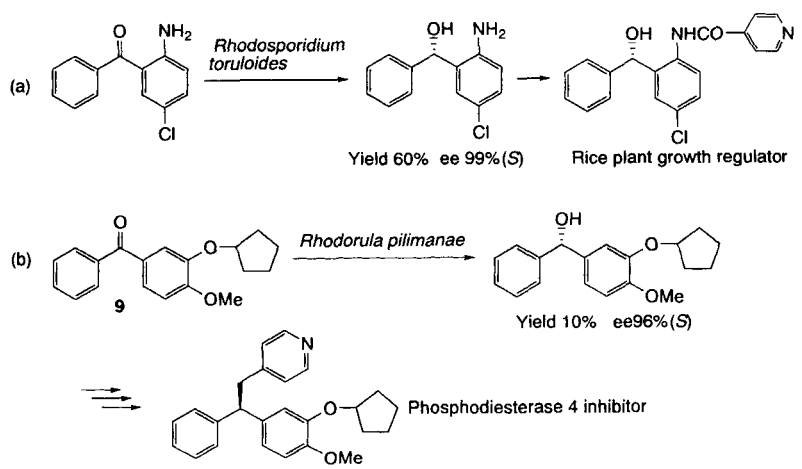


Figure 15-42. Reduction of diaryl ketones for the synthesis of bioactive compounds[243, 244].

15.1.5.7
Diastereoslective Reductions (Dynamic Resolution)

Enantio and diastereoselective reduction (dynamic resolution) of keto esters and ketones can be achieved using yeast and other microorganisms[55, 70, 74, 245–253]. As shown in Fig. 15-43, when the racemization rate of the keto ester is faster than that for the yeast reduction, and the product hydroxyester is not racemized under the reaction conditions, then the yeast reduction may proceed enantioselectively and

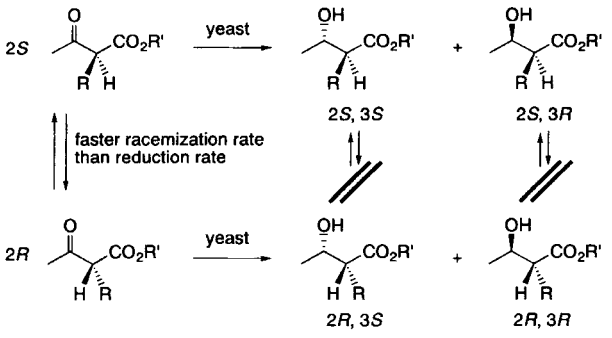


Figure 15-43. Diastereoselective reduction.

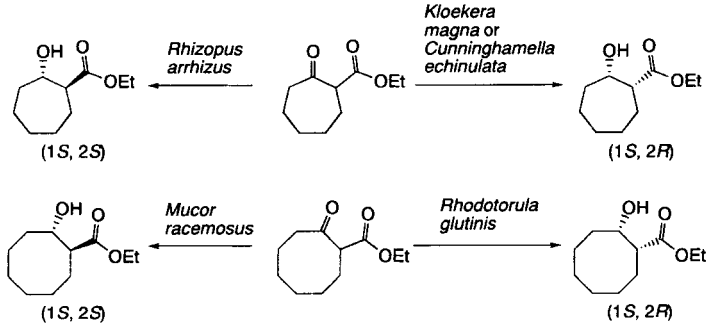


Figure 15-44. Diastereoselective reduction of cyclic keto esters[245].

diastereoselectively; thus only one stereoisomer out of the four possible ones can be obtained in one step. Actually, when bakers' yeast was used for the reduction of neopentyl 2-methyl-3-oxobutanoate (R = Me, R' = neopentyl), then the ratio of (2*R*, 3*S*) : (2*S*, 3*R*) : (2*S*, 3*S*) : (2*R*, 3*R*) products was found to be 96 : < 1 : 4 : < 1[247]. When an enzyme was isolated from the yeast, then the diastereoselectivity was improved to > 99 : 1, and only a single isomer was obtained[248]. Another example is the large scale reduction of ethyl 2-methyl-3-oxobutanoate by *Klebsiella pneumoniae* IFO 3319[70]. On a 200 L scale, 2 Kg of the substrate were converted into the (2*R*, 3*S*)-hydroxyester with 99% de, > 99% ee, and 99% chemical yield as shown in Table 15-2.

Enantio- and diastereoselective reduction of cyclic keto esters are also achieved using various microorganisms (Fig. 15-44)[245]. By selecting a suitable organism, *syn*- and *anti*-hydroxyesters may be synthesized enantio- and diastereoselectively.

15.1.5.8
Chemo-enzymatic Synthesis of Bioactive Compounds

Ketones with various functionalitis, containing F, Cl, N, S, O, *etc.*, have been shown to be reduced by a biocatalyst, and by using the biocatalytic reduction as a key step, the chemoenzymatic synthesis of many bioactive compounds have been re-

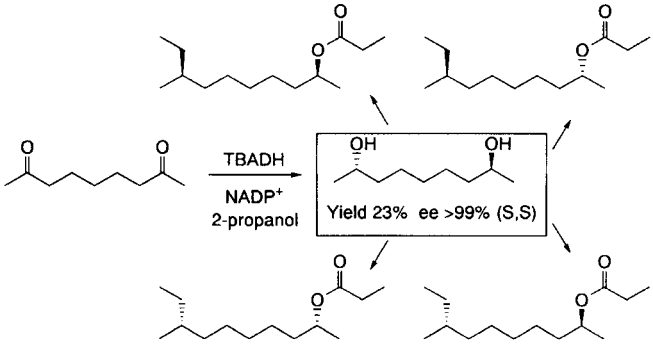


Figure 15-45. Synthesis of all four isomers of the western corn rootworm sex pheromone[234].

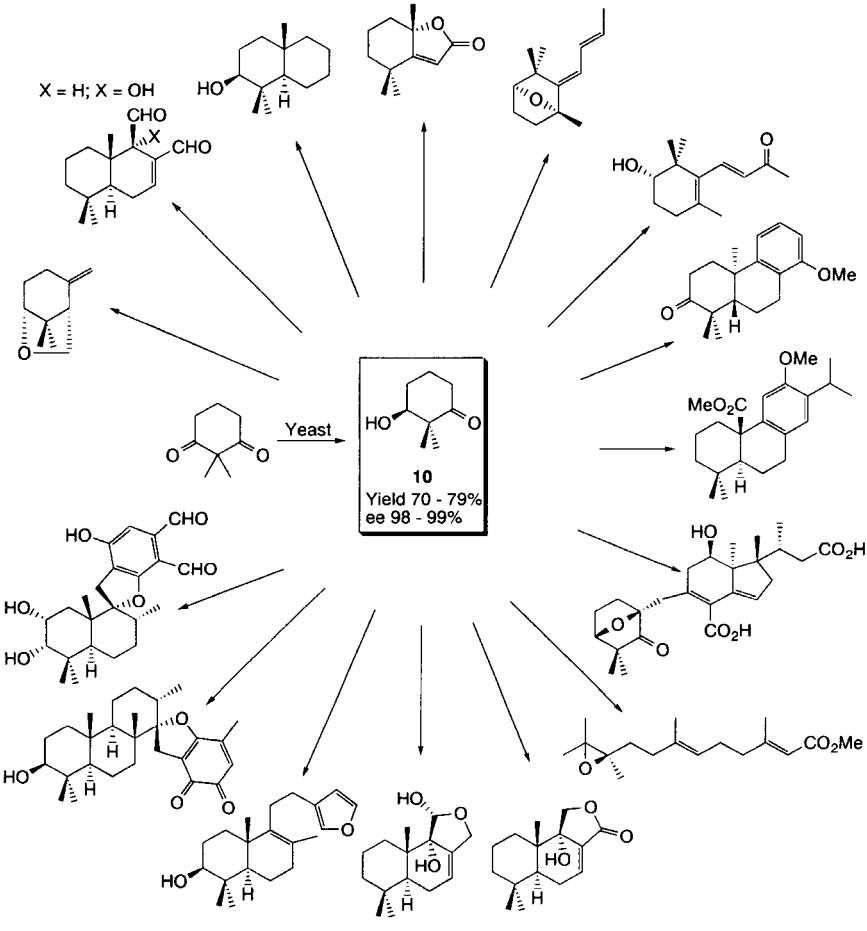


Figure 15-46. Synthesis of natural products from a key intermediate obtained by yeast reduction.

ported[122, 129, 199, 228–230, 234, 235, 243, 254–274]. For example, 2,8-nonandione can be reduced enantioselectively by TBADH to furnish the corresponding diol, from which all four isomers of 8-methyldec-2-yl propanoate, the western corn rootworm sex pheromone, were prepared (Fig. 15-45)[234].

One of the most versatile key intermediates discovered to date is the hydroxyketone **10** which is synthesized by the yeast reduction of the corresponding diketone[229, 230]. Starting with **10**, many terpenes have been enantioselectively synthesized by Mori *et al.*, as shown in Fig. 15-46.

15.2 Reduction of Various Functionalities

Kaoru Nakamura and Tomoko Matsuda

15.2.1 Reduction of Aldehydes

Many aldehyde reductases transform both aldehydes and ketones[138, 144, 275, 276]. For example, phenylacetaldehyde reductase from a styrene-assimilating *Corynebacterium* strain, ST-10, reduces aldehydes and ketones as shown in Table 15-18[138]. Other aldehyde reductases such as one from *Sporobolomyces salmonicolor* also reduce aldehydes as well as ketones[144, 275].

Organometallic aldehydes can be reduced enantioselectively with dehydrogenases. For example, optically active organometallic compounds having planar chiralities were obtained by biocatalytic reduction of racemic aldehydes with yeast[277, 278] or HLADH[279] as shown in Fig. 15-47.

The dynamic resolution of an aldehyde is also possible as shown in Fig. 15-48[280]. The racemization of the starting aldehyde and enantioselective reduction of a carbonyl group by bakers' yeast resulted in the formation of tertiary chiral carbon centers. The ee of the product was improved from 19% to 90% by changing the ester moiety from the isopropyl group to the neopentyl group.

Table 15-18. Examples of substrates of phenylacetaldehyde reductase from *Corynebacterium* strain, ST-10[138].

Substrate (m*M*) (aldehyde)	Relative activity (%)	Substrate (m*M*) (ketone)	Relative activity (%)
Acetaldehyde (3)	0	Acetone (3)	0
n-Valeraldehyde (3)	181	2-Hexanone (3)	207
n-Hexyl aldehyde (3)	1220	2-Heptanone (3)	760
Phenylacetaldehyde (3)	100	Acetophenone (3)	35
3-Phenylpropionaldehyde (1)	364	4-Phenyl-2-butanone (3)	29

ported[122, 129, 199, 228–230, 234, 235, 243, 254–274]. For example, 2,8-nonandione can be reduced enantioselectively by TBADH to furnish the corresponding diol, from which all four isomers of 8-methyldec-2-yl propanoate, the western corn rootworm sex pheromone, were prepared (Fig. 15-45)[234].

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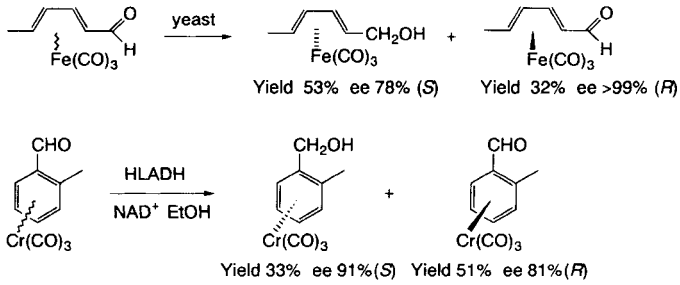


Figure 15-47. Reduction of organometallic aldehydes to produce alcohols with planar chiralities[277–279].

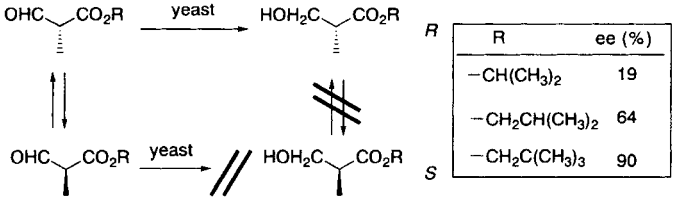


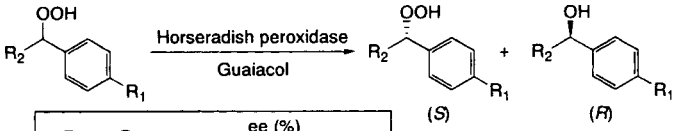
Figure 15-48. Reduction of aldehyde with dynamic resolution[280].

15.2.2
Reduction of Peroxides to Alcohols

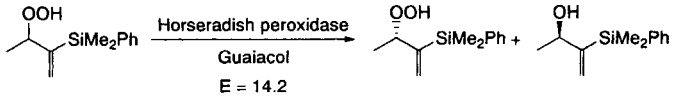
Horseradish peroxidase has been used for the reduction of peroxide to alcohol[281–284]. The enzyme selectively recognizes sterically uncumbered *(R)*-alkyl aryl hydrogenperoxides, which allows kinetic resolution to provide *(R)*-alcohol and *(S)*-peroxide. However, poor enzyme recognition is observed with hydroperoxides possessing larger R_2 groups such as a propyl or an isopropyl moiety as shown in Fig. 15-49. This reaction can be performed on a preparative scale conveniently to provide optically pure hydroperoxides.

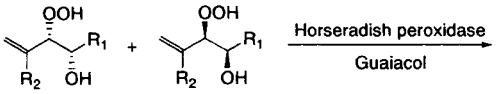
15.2.3
Reduction of Sulfoxides to Sulfides

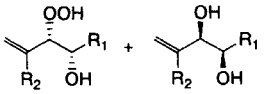
Asymmetric synthesis of sulfoxides can also be achieved by biocatalytic reduction. One example is the reduction of alkyl aryl sulfoxides by intact cells of *Rhodobacter sphaeroides f. sp. denitrificans*[285]. In the reduction of methyl *p*-substituted phenyl sulfoxides, *(S)*-enantiomers were exclusively deoxygenated while enantiomerically pure *(R)*-isomers were recovered in good yield. For poor substrates such as ethyl phenyl sulfoxide, the repetition of the incubation after removing the toxic product was effective in enhancing the ee of recovered *(R)*-enantiomers to 100 % as shown in Table 15-19.



R_1	R_2	ee (%) (-)-(*S*)-ROOH	(+)-(*R*)-ROH
H	Me	>99	>99
Cl	Me	>95	>95
H	Et	93	95
H	Pr	<5	<5
H	*i*-Pr	15	14
etc.			



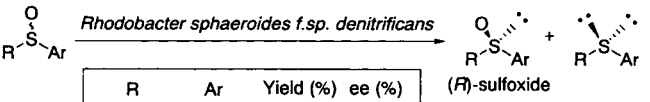




R_1	R_2	E
Me	H	10
Et	H	>200
i-Pr	H	>200
t-Bu	H	30
Me	Me	2

Figure 15-49. Reduction of peroxides to alcohols[281–284].

Table 15-19. Reduction of sulfoxide to obtain optically pure (*R*)-sulfoxide[285].



R	Ar	Yield (%)	ee (%)
Me	Ph	46	100
Me	*p*-Me-C_6H_4	40	100
Me	*p*-Br-C_6H_4	43	100
Me	*p*-MeO-C_6H_4	47	>99
Me	$PhCH_2$	41	90
Et	Ph	41	100
n-Pr	Ph	54	21

15.2.4
Reduction of Azide and Nitro Compounds to Amines

Bakers' yeast catalyzes the reduction of azides and nitro compounds to amines[286–291]. For example, it catalyzes chemoselective reduction of azidoarenes to arenamines as shown in Fig. 15-50[286, 287]. Excellent yields are obtained for various aromatic compounds on reaction at room temperature. Aromatic nitro compounds

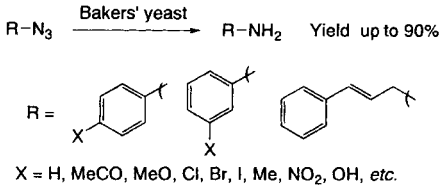


Figure 15-50. Reduction of azide to amine by bakers' yeast[286, 287].

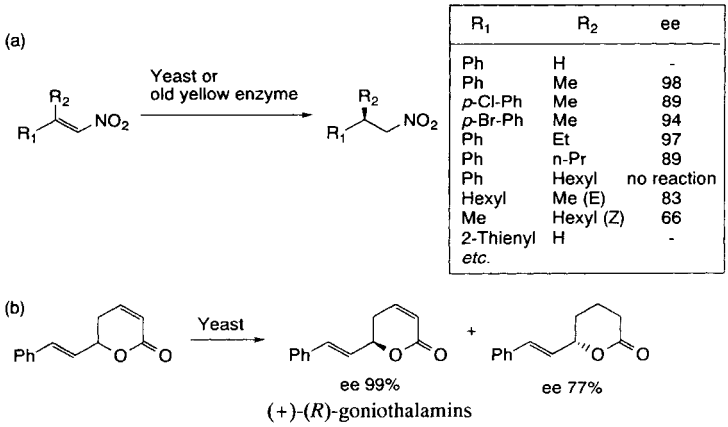
containing *o*-, *m*-, or *p*-electron withdrawing groups, such as carbonyl, halogen and nitro, were also selectively and rapidly reduced to their corresponding amino derivatives in good yields using bakers' yeast in basic solution[288].

N-oxides can also be reduced. For example, the microbial deoxygenation of a series of aromatic and heteroaromatic *N*-oxide compounds, including quinoline *N*-oxides, isoquinoline *N*-oxides, 2-aryl-2*H*-benzotriazole 1-oxides, benzo[c]cinnoline *N*-oxide and azoxybenzene, has performed with bakers' yeast–NaOH to afford quinolines and pyridines[291].

15.2.5
Reduction of Carbon-Carbon Double Bonds

The reduction of carbon–carbon double bonds to single bonds has been studied with various substrates[51, 124, 292–306]. For example, Ohta *et al.* demonstrated that the reduction of a number of 1-nitro-1-alkenes by fermenting bakers' yeast was enantioselective, resulting in the formation of optically active 1-nitroalkanes as shown in Fig. 15-51(a)[294, 295]. On the other hand, Fuganti *et al.* reduced α,β-unsaturated-δ-lactones to produce enantiomerically pure (+)-(*R*)-goniothalamins [Fig. 15-51(b)], which show CNS activity. They also performed the kinetic resolution of the corresponding embryotoxic epoxide with yeast[296].

One of the most studied enzymes for the reduction of carbon–carbon double



R_1	R_2	ee
Ph	H	-
Ph	Me	98
p-Cl-Ph	Me	89
p-Br-Ph	Me	94
Ph	Et	97
Ph	n-Pr	89
Ph	Hexyl	no reaction
Hexyl	Me (E)	83
Me	Hexyl (Z)	66
2-Thienyl	H	-
etc.		

Figure 15-51. Examples of substrate specificity of yeast reduction of olefins[294–296].

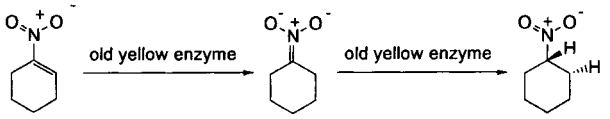


Figure 15-52. Mechanism of the reduction of nitro olefin by "old yellow enzyme" from yeast[292–294].

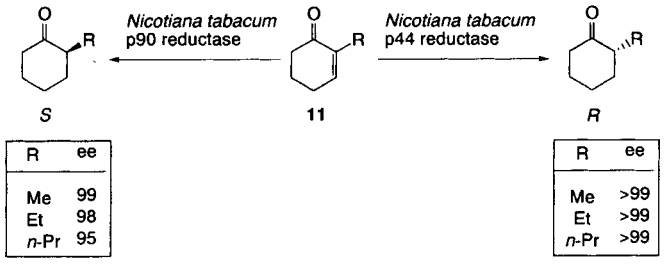


Figure 15-53. Reduction of carbon–carbon double bonds by reductases from plant cell culture[298].

bonds is the "old yellow enzyme" from yeast[292–294] which has been shown efficiently to catalyze the NADPH-linked reduction of nitro olefins. The reduction of the nitro-olefin proceeds in a stepwise fashion (Fig. 15-52). The first step involves hydride transfer from the enzyme-reduced flavin to the β-carbon of the nitro-olefin which forms a nitronate intermediate that is freely dissociable from the enzyme. The second step, protonation of the nitronate at the α-carbon to form the final nitroalkane product, is also catalyzed by the enzyme.

Photosynthetic microorganisms and plant cell cultures are very important sources of enzymes for the reduction of olefins[51, 298]. For example, Hirata *et al.* found that reduction of enone **11** with *Nicotiana tabacum* p90 reductase and *Nicotiana tabacum* p44 reductase affords *(S)*- and *(R)*-alkylcyclohexanones, respectively, with excellent enantioselectivities as shown in Fig. 15-53. They also found two enone reductases from *Astasia longa*, a nonchlorophyllous cell line classified in *Euglenales*, and studied the mechanism. Both catalyzed enantiospecific *trans*-addition of hydrogen atoms to carvone from the *si*-face at the α-position and from the *re*-face at the β-position.

15.2.6
Transformation of α-Keto Acid to Amine

A dehydrogenase can also be used for the transformation of an α-keto acid to an amine (Fig. 15-54). The chiral intermediate for an antihypertensive drug was prepared by reduction of an α-keto acid with glutamate dehydrogenase from beef liver. The cofactor NADH was regenerated using glucose dehydrogenase from *Bacillus* sp.[307].

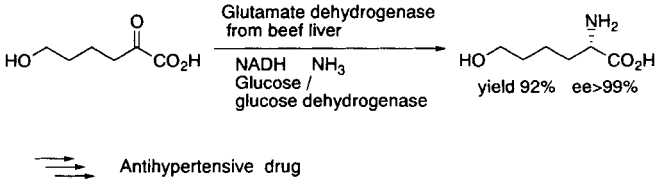


Figure 15-54. Reduction of α-keto acid to amine[307].

15.2.7
Reduction of Carbon Dioxide

15.2.7.1
Reduction of CO_2 to Methanol

Syntheses using CO_2 as a carbon source are attracting growing interest. The development of environmentally benign methods to utilize CO_2 is very important due to the abundance of CO_2. For this purpose, dehydrogenases have been successfully utilized. Formate, formaldehyde and alcohol dehydrogenases are used for the reduction of CO_2 to methanol as shown in Fig. 15-55[33, 308–311].

For the efficient production of methanol, electrochemical methods have been used (Fig. 15-55)[33, 308, 310, 311]. Electrochemically, CO_2 was converted into formate by formate dehydrogenase with the aid of methyl viologen or pyrroloquinolinequinone as a mediator. Methanol dehydrogenase was used to reduce formate to formaldehyde and methanol with the same system[308, 310, 311].

An approach for the conversion of CO_2 into formic acid which combines a semiconductor photoelectrode with formate dehydrogenase is very interesting[33]. Electrons in the semiconductor can be produced with light of wavelengths shorter than 900 nm. Then, the photogenerated electrons were transferred to CO_2 through methyl viologen to produce formic acid as shown in Fig. 15-56[33].

Another highly efficient process involves the immobilization of three enzymes in a silica sol-gel[309]. Since the process consists of a sequential reaction of *in situ* generated substrates with three different enzymes, the confinement of the system in a porous matrix resulted in an enhanced probability of the reactions as shown in Fig. 15-57 due to an overall increase in local concentration of reactants within the nanopores of the sol-gel processed glasses[309].

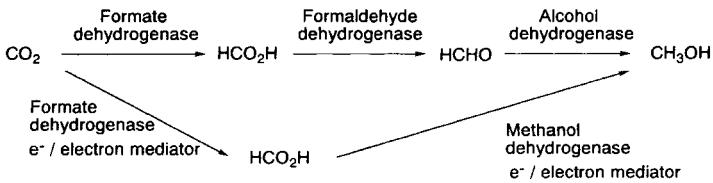


Figure 15-55. Reduction of CO_2 to methanol with dehydrogenases.

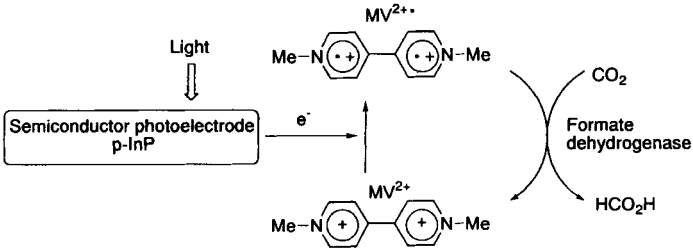


Figure 15-56. Photoelectrochemical pumping of enzymatic CO_2 reduction[33].

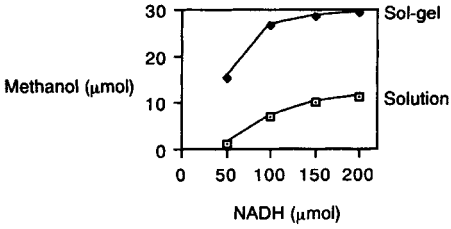


Figure 15-57. Effect of the confinement of the three enzymes in a porous matrix on methanol production[309].

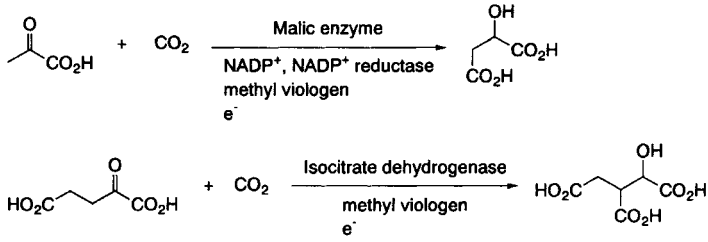


Figure 15-58. Electrochemical reductive fixation of CO_2[312, 313].

15.2.7.2
Reductive fixation of CO_2

Reductive fixation is an another important process. Malic enzyme and isocitrate dehydrogenase catalyze both the reduction of the carbonyl group in an α-keto acid and fixation of CO_2 at the α-position with the aid of an electric power source and an electron mediator (Fig. 15-58)[312, 313]. Uniquely, the reaction using isocitrate dehydrogenase does not require the use of $NADP^+$. When CO_2 is reductively fixed in an organic molecule, the enzyme is oxidized; the oxidized enzyme is ultimately reduced back to its original form by methyl viologen cation radicals[312].

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15.3 Reduction of C=N bonds

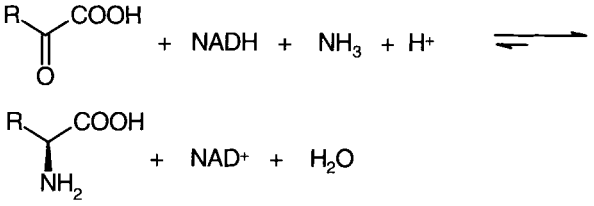
Andreas S. Bommarius

15.3.1 Introduction

Enantiospecific reduction of C=N bonds is of interest for the synthesis of α-amino acids and derivatives such as amines. While nonenzymatic reductive amination has been known since 1927[1], only recently have enzymatic procedures to L-amino acids became established. The reduction can be achieved by different enzymes following different mechanisms, e.g. by pyridoxalphosphate (PLP)-dependent transaminases (E.C. 2.6.1, discussed in Chapter 12.7) or by amino acid dehydrogenases (E.C. 1.4.1) using NADH or NADPH as the cofactor. The synthetic usefulness of the transaminase reaction is diminished by the location of the equilibrium (K_{eq} often is close

to one), so that complex mixtures result, which are often laborious to separate (for solutions to this problem, see Chapter 12.7). For this reason, this chapter focuses on the reduction of C=N bonds by reductive amination with amino acid dehydrogenases, AADHs.

Reductive amination of α-keto acids to α-amino acids is similar to the reduction of C = O bonds to the corresponding α-hydroxy acids. In an equilibrium reaction, α-keto acids can be reductively aminated to α-amino acids or, *vice versa*, α-amino acids can be oxidatively deaminated:



(1)

A very promising process route is the reductive amination of prochiral α-keto acids to α-amino acids with AADHs and the cofactor NADH and its regeneration by co-oxidation of formate to CO_2 by formate dehydrogenase (Fig. 15.3-1).

This asymmetric synthesis route possesses a number of advantages rendering it attractive in today's context of seeking environmentally benign processes:

- compact synthesis of α-keto acid substrates,
- formation of harmless and easily separable CO_2 as the only co-product,
- extreme enantioselectivity of amino acid dehydrogenases, and
- yields of up to 100% with respect to α-keto acid, resulting in no undesirable enantiomers and other by-products.

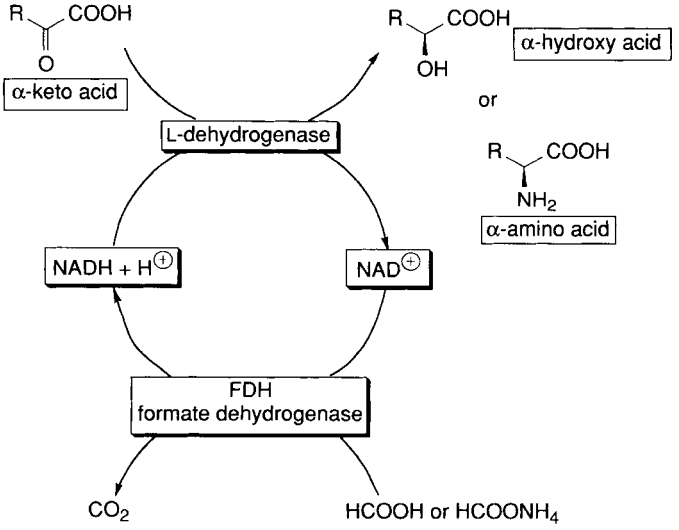


Figure 15.3-1. Schematic of enzymatic reductive amination with cofactor regeneration.

Table 15.3-1. List of NAD(P)$^+$-dependent amino acid dehydrogenases[5].

E.C. Number	Enzyme	Coenzyme	Sourcea
1.4.1.1	Alanine DH	NAD$^+$	B (*Bacillus, Streptomyces, Halobacterium*)
1.4.1.2	Glutamate DH	NAD$^+$	B, F, Y, P
1.4.1.3	Glutamate DH	NAD(P)$^+$	A, F, *Tetrahymena*
1.4.1.4	Glutamate DH	NAD$^+$	B, F, Y, *Chlorella*
1.4.1.7	Serine DH	NAD$^+$	P
1.4.1.8	Valine DH	NAD(P)$^+$	B (*Alcaligenes, Streptomyces*), P
1.4.1.9	ucine DH	NAD$^+$	B (*Bacillus, Clostridium*)
1.4.1.10	Glycine DH	NAD$^+$	B (*Mycobacterium*)
1.4.1.11	3,5-Diaminohexanoate DH	NAD$^+$	B (*Clostridium*)
1.4.1.12	2,4-Diaminopentanoate DH	NAD$^+$	B (*Clostridium*)
1.4.1.15	Lysine DH	NAD$^+$	Human, B (*Agrobacterium*)
1.4.1.16	Diaminopimelate DH	NADP$^+$	B (*Bacillus, Corynebacterium*)
1.4.1.20	Phenylalanine DH	NAD$^+$	B (*Brevibacterium, Bacillus, Rhodococcus*)
1.4.1.-	Tryptophan DH	NAD(P)$^+$	P

a Abbreviations: B: bacterium; F: fungi; Y: yeast; A: animal; P: plant; DH: dehydrogenase

With three exceptions (AlaDH from *Phormidium lapideum*, L-lysine-ε-dehydrogenase and meso-α,ε-diaminopimelate DH) all of the AADHs (Table 15.3-1) catalyze reduction of prochiral keto acids to the L-amino acids [(S)-configuration]. The natural function of L-AADHs is not known. The D-AADHs that have been found appear to be iron-sulfur membrane-associated flavoenzymes which seem to catalyze the oxidative reaction from keto acids to amino acids only; artificial dyes and the coenzyme Q analog serve as electron acceptors but not oxygen[2–4]. AADHs have been screened from a variety of organisms (Table 15.3-1), the most important enzymes for synthesis are alanine dehydrogenase (AlaDH, E.C. 1.4.1.1), phenylalanine dehydrogenase (PheDH, E.C. 1.4.1.20), and particularly leucine dehydrogenase (LeuDH, E.C. 1.4.1.9). The ubiquitous glutamate dehydrogenase (GluDH, E.C. 1.4.1.2.–4), however, is still the most studied member of the group.

Reviews on AADHs: Apart from early review articles on individual amino acid dehydrogenases by Schütte et al. (1985; LeuDH from *B. cereus*)[6], Ohshima et al. (1985a; LeuDH from *B. species*)[7] and Hummel et al. (1987; PheDH from *Rh. rhodocrous*)[8], comprehensive reviews have been published by Hummel and Kula (1989)[9], Ohshima and Soda (1989 and 1990)[5,10,11] and by Brunhuber and Blanchard (1994)[12].

15.3.2
Structural Features of Amino Acid Dehydrogenases (AADHs)

Most of the AADHs possess hexameric structure, although octamers, tetramers, dimers and even monomers have been found. The subunits are usually of similar size: for instance, most bacterial AADHs are hexamers with a molecular weight of around 49 000 per subunit.

Table 15.3-2. Identities of protein sequences of different amino acid dehydrogenases (in per cent) [22]. The data were calculated via BLAST search in the database 'Swissprot' [23].

Protein	LeuDH, *B. cereus*	LeuDH, *B. sphaericus*	PheDH, *Rh. rhodocrous*	PheDH, *Th. intermedius*	GluDH, *C. symbiosum*
LeuDH, *B. stearothermophilus*	82.5	79.9	32.0	45.6	12.6
LeuDH, *B. cereus*	–	76.9	31.5	44.5	13.4
LeuDH, *B. sphaericus*		–	31.7	41.8	14.0
PheDH, *Rh. rhodocrous*			–	26.4	12.4
PheDH, *T. intermedius*				–	14.2

15.3.2.1
Sequences and Structures

Several amino acid dehydrogenases have been screened from a variety of microorganisms, the preparatively most important are phenylalanine dehydrogenase (PheDH, from *Rhodococcus* sp. M4) and leucine dehydrogenase (LeuDH, from *Bacillus stearothermophilus* and *Bacillus cereus*). As of the end of February 2001, more than 20 gene and protein sequences for AADHs except GluDH (which more than triples the number) and 3D crystal structures from five different AADHs have been deposited (GluDH from *Clostridium symbiosum* [13], LeuDH from *B. sphaericus* [14], AlaDH from *Phormidium lapideum* [15], PheDH from *Nocardia* sp 239 [16] and PheDH from *Rhodococcus* sp. M4 [17,18]). Sequence homologies and similarities of 3D structures of the members of several organisms are so high that amino acid dehydrogenases can be termed a single superfamily, generated through divergent evolution [19–21] (Table 15.3-2).

Remarkable, on one hand, is the high degree of identity of the three leucine dehydrogenases, and on the other hand the sequence of glutamate dehydrogenase, which bears no homology to the other dehydrogenases. Although overall sequence homology varies from around 20% up to 80%, the residues essential for the three-dimensional structure of a subunit, for nicotinamide cofactor binding, and for catalysis have been conserved [20]. While a complex between NAD^+ and GluDH from *Clostridium symbiosum* left the overall conformation unaltered [24], a drastic conformational change (hinge movement) was observed on binding of the glutamate [13].

15.3.3
Thermodynamics and Mechanism of Enzymatic Reductive Amination

15.3.3.1
Thermodynamics

For reductive amination, basically no thermodynamic limitation exists: for the leucine/ketoleucine reaction at pH 11.0, K_{eq} equals 9×10^{12} [25], for phenylalanine/phenylpyruvate at pH 7.95 a K_{eq} of 2.5×10^{7} has been reported [18], thus, the maximum degree of conversion is very close to 100%. Coupling of the reductive amination reaction with cofactor regeneration via the FDH/formate reaction, which is irreversible, further helps to pull the equilibrium towards the amino acid product.

15.3.3.2
Mechanism, Kinetics

As will be elucidated below, the mechanism of reductive amination and the geometry of the active center[13, 18, 19, 26, 27] cause the (*S*)-configured amino acid products of the reaction to be completely enantiomerically pure, an important criterion for a large-scale application.

The catalytic mechanism of AADHs has been studied most thoroughly with GluDH from *C. symbiosum*[13,24] and with PheDH from *Rhodococcus* M4[18]. The mechanism was found to be remarkably similar in both cases so that the prediction by Stillman et al.[13] seems to have been borne out. In Fig. 15.3-2, the study on PheDH is illustrated[18]:

Following the scheme in Fig. 15.3-2, which depicts oxidative deamination, in a clockwise fashion starting from the top left, the α-N-protonated L-Phe molecule is stabilized by the ε-group of Lys66 at the carbonyl group as well as by the ε-group of Lys78 via a water molecule, the carbonyl group of Pro117 and the β-carboxyl group of Asp118 at the α-amino group. The first intermediate is the protonated imine after steps (2) and (3) in which Lys78 picks up the proton from the α-N-group of L-Phe and delivers a hydrogen to the *Si* face of the cofactor NAD^+ with deprotonation of Lys78. Accompanied by another Lys78 protonation, the water molecule adds to the imine

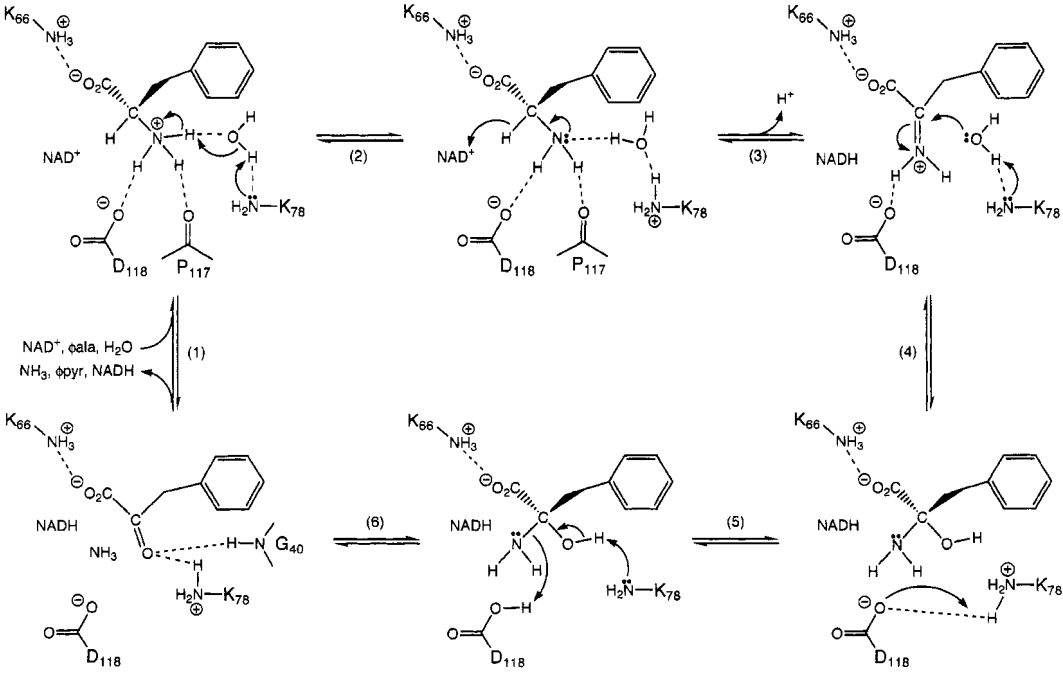


Figure 15.3-2. Proposed mechanism for amino acid dehydrogenases (with PheDH as an example)[18].

carbon to form the carbinolamine, the second intermediate [step (4)]. The Lys78 proton is picked up by Asp118 [step (5)] and in turn by the amino group [step (6)] of the substrate to liberate NH_3 and with the formation of phenylpyruvate. The keto group is stabilized by the protonated ε-sidechain of Lys78 as well as a by a proton from Gly40. The positioning of Lys78 and Gly40 also prevents the oxidation of phenyllactate, so that PheDH cannot act as a HicDH.

A similar mechanism had already been proposed for GluDH from *C. symbiosum*[13]; the only major difference seems to be the attribution of the initial deprotonation of the amino acid molecule to Asp165 (which corresponds to Asp117 on PheDH) instead of Lys125 (Lys78 in PheDH). The Lys125 in GluDH is known to have a low p*K* value[28], which causes this residue to act as a proton shuttle more easily.

The optimum degree of protonation and catalytically important amino acid residues can be determined from a log V_{max}-pH diagram[29]: on the acidic and alkaline side of the optimum pH, log V_{max} decreases nearly linearly with pH, the two slopes intersect at the optimum degree of protonation, which is also the optimum point of activity. The experimentally observed optimum pH value of 9.2–9.3 for LeuDH[30], corresponding to two p*K* values of around 8.7 and 10.0 for amino acid residues participating in the catalytic step, can be linked to lysine residues, corroborating the results of Rife and Cleland (1980)[26] and Sekimoto et al. (1993)[27] for the case of GluDH. Brunhuber et al. in their study of PheDH assigned their pK_as values of 8.1 and 9.4 to Asp118 and Lys78, respectively[18]. The influence of pH on reductive aminations with AADHs can also be explained by the dissociation equilibrium of ammonia (pK_a value 9.25). Only an uncharged ammonia molecule can be accepted by LeuDH[26, 30] so that a minimal pH of around 7.5 has to be kept throughout the reaction.

15.3.4
Individual Amino Acid Dehydrogenases

15.3.4.1
Leucine Dehydrogenase (LeuDH, E.C. 1.4.1.9)

Isolation and characterization of LeuDH has been pioneered by Hummel et al.[31] (from *B. sphaericus*), Schütte[6] (from *B. cereus*), and by Ohshima and Soda (from mesophilic *Bacillus sphaericus* and from moderately thermophilic *Bacillus stearothermophilus*[10, 20, 32]). The biochemical data for the last two enzymes, however, do not differ much, as Table 15.3-3 reveals.

The LeuDH from *B. stearothermophilus* as compared with the *B. sphaericus* enzyme has an extended pH range of activity (5.5–10 vs. 6.5–8.5), a higher heat stability (70 vs. 50 °C after a heat treatment of 5 min), a longer half-life (several months vs. six days at pH 7.2 and 6 °C), and much greater stability against organic solvents and denaturants[10].

LeuDH from *B. stearothermophilus* had already been cloned and overexpressed[20, 33] during early studies. Recently, the production of recombinant enzyme from *B. cereus* even on a large scale has been demonstrated[34, 35].

Table 15.3-3. Properties of LeuDH from *Bacillus sphaericus* and *Bacillus stearothermophilus*[10].

Source		*B. sphaericus*	*B. stearothermophilus*
M_r (kDa)		245 000	300 000
Subunit (M_r)		41 000	49 000
		hexamer	hexamer
Optimum pH:	deamination	10.7	11.0
	amination	9.0–9.5	9.0–9.5
Coenzyme		NAD (K_M 0.39 mM)	NAD (K_M 0.49 mM)
Substrate specificity (in % of L-leucine)			
Deamination:	L-leucine	100 (K_M 1.0 mM)	100 (K_M 4.4 mM)
	L-valine	74 (1.7)	98 (3.9)
	L-isoleucine	58 (1.8)	73 (1.4)
	L-norvaline	41 (3.5)	–
	L-α-aminobutyrate	14 (10)	–
	L-norvaline	10 (6.3)	–
	D-leucine	0	0
Amination:	α-ketoisocaproate	100 (0.31)	100
	α-ketoisovalerate	126 (1.4)	167
	α-ketovalerate	76 (1.7)	86
	α-ketobutyrate	57 (1.7)	45
	α-ketocaproate	46 (7.0)	–

The substrate specificity of LeuDHs, catalyzing mainly branched-chain α-keto acids to the α-amino acids, has been investigated by Zink and Sanwal (1962)[36] and subsequently by Schütte et al. (1985: *B. cereus*)[6], Ohshima and Soda (1989; *Bacillus stearothermophilus* and *Bacillus sphaericus*)[5], Nagata et al. (1990; *Bacillus DSM 7330*)[37], Misono et al. (1990; *Corynebacterium pseudodiphtheriticum*)[38] and by Bommarius et al. (1994; *Bacillus stearothermophilus*)[39]. In addition to the proteinogenic amino acids valine, leucine, and isoleucine, unnatural amino acids such as *tert*-leucine[40] or L-β-hydroxy-valine[41] can be synthesized.

The kinetic parameters of several leucine dehydrogenases show a similar pH-profile. The opposite tendency of V_{max} and K_M for all substrates is remarkable: the dimethyl-substituted substrates show K_M values above 10 mM (V_{max} values are between 0.2 and 30% of the reactivity of 2-oxo-4-methyl-pentanoic acid, the base case), whereas K_M values below 1 mM are typical for good substrates ($V_{max} \approx 100\%$ compared with the base case, 2-oxo-4-methyl-pentanoic acid).

15.3.4.2
Alanine Dehydrogenase (AlaDH, E.C. 1.4.1.1)

AlaDH has been isolated and characterized from both mesophilic (*B. subtilis* and *B. sphericus*)[42] and thermophilic (*B. stearothermophilus*)[43] organisms. For cloning and purification of AlaDH, see ref.[44]. The narrow substrate specificity of AlaDH[42] renders the enzyme useful for synthesis of L-alanine and analogs only, such as [^{15}N]-L-alanine[45], 3-fluoro-L-alanine[46], and 3-chloro-L-alanine[47].

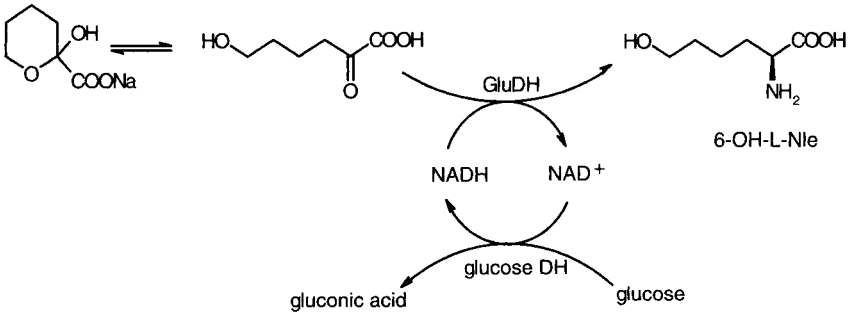


Figure 15.3-3. Synthesis of 6-hydroxy-L-norleucine with GluDH/glucose DH[51].

15.3.4.3

Glutamate Dehydrogenase (GluDH, E.C. 1.4.1.2–4)

GluDH has been investigated by the groups of Engel and Rice since the 1980s so that more is known about GluDH, especially from *C. symbiosum*, than about any other AADH. Although there is no sequence identity to other AADHs beyond random similarity (Table 15.3-2), site-directed mutagenesis of two amino acids residues, K89L and S380V, led to similar activity levels towards glutamate, norleucine and methionine and demonstrated the importance especially of the K89L mutation[48, 49]. Studies on GluDH from the same source define the knowledge base regarding conformational change of the enzyme upon binding of the substrate but not upon the preceding binding of the cofactor. These conformational changes also seem to be responsible in part for substrate specificity[50].

Just as with other AADHs, GluDH has potential as a catalyst in synthesis: beef liver GluDH was the best catalyst for the reductive amination of 2-keto-6-hydroxyhexanoic acid Na salt to 6-hydroxy-L-norleucine, a potentially important building block for the vasopeptidase Vanlev (BMS) (Fig. 15.3-3)[51]. The reaction of 95 mM substrate (2 : 1 mixture of 2-keto-6-hydroxy-hexanoic acid Na salt in equilibrium with 2-hydroxy-tetrahydropyran-2-carboxylic acid) was complete in 3 h, resulting in an amino acid product of 89–92% chemical yield and >99% optical purity. As the keto acid substrate is very cumbersome to synthesize, an alternative way of providing the keto acid substrate was the separation of D,L-6-hydroxynorleucine, which can be prepared easily from 4-hydroxybutylhydantoin, by D-amino acid oxidase to L-amino acid and keto acid where the latter in turn was reduced by GluDH/NADH[51]. Both FDH/formate and glucose DH/glucose were employed for cofactor regeneration.

15.3.4.4

Phenylalanine Dehydrogenase (PheDH, E.C. 1.4.1.20)

An enzyme catalyzing the reductive amination of phenylpyruvate to the desired L-Phenylalanine was first found by Hummel et al.[52] in a strain of *Brevibacterium* and later in *Rhodococcus* sp.[8, 53]. Table 15.3-4 summarizes the microbiological and kinetic data[9].

Table 15.3-4. Comparison of PheDH from *Brevibacterium* and *Rhodococcus* species[9].

Parameter	*Brevibacterium*	*Rhodococcus*
Microbiological data:		
enzyme yield (U L^{-1}) after addition of 1 % of		
L-phenylalanine	210	15 200
L-histidine	120	1800
L-phenylalaninamide	-	3500
L-isoleucine	0	0
D-phenylalanine	204	0
DL-phenylalanine	214	0
	9.0	9.25
Enzymological data:		
pH optimum		
reductive amination		
oxidative deamination	10	10
	0.11	0.16
K_M (mM)		
phenylpyruvate		
p-hydroxypyruvate	0.24	2.4
indolepyruvate	8.0	7.7
2-oxo-4-methylmercaptobutyrate	3.0	2.1
	100	100
V_{max} (relative to phenylpyruvate)		
phenylpyruvate		
p-hydroxypyruvate	96	5
indolepyruvate	24	3
2-oxo-4-methylmercaptobutyrate	59	33
	47	130
K_M (μM) NADH		
K_M (mM) NH_4^+	431	387
	4–8 h	10 d
Stability:		
stored at 4 °C ($t_{1/2}$)		
deactivation (% d^{-1}) under operation	26	5
Reference	8	53

Table 15.3-5. Substrate specifity of different PheDHs[39].

Substrate[a]	*Rhodococcus rhodocrous* V_{max} (U m L^{-1})	K_M (mM)	Rel. activity (%)	*B. sphaericus* Rel. activity (%)
Ketoisocaproate			4.2	
Keto-methionine[b]	50	2.1	33	6.0
Phenylpyruvate	**150**	**0.16**	**= 100**	**= 100**
p-OH-phenylpyruvate[b]	7.5	2.4	5	138
Indolepyruvate[b]	4.5	7.7	3	n.d.[c]
Keto-4-phenylbutyrate	96	0.01	64	1.9
Keto-5-phenylvalerate	46	0.65	30	1.5

a Conditions: pH 8.0, T = 25 °C, [S] = 0.1 M; comparison: LeuDH from *B. cereus*: 2-oxo-4-methyl-pentanoic acid = 100 %, 2-oxo-4-phenylbutyrate = 0.2 %; *B. sphaericus* data from[55]
b As in a except for a pH of 8.5[8]
c Not determined

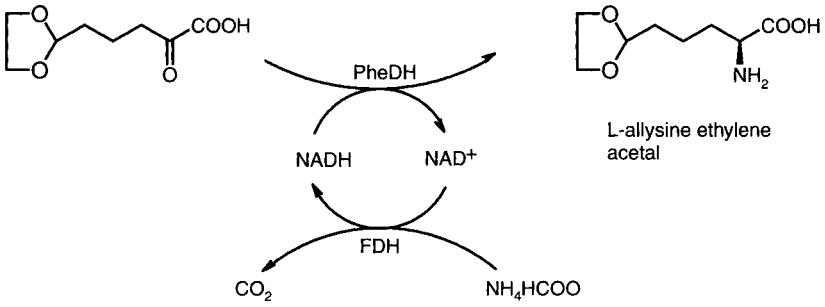


Figure 15.3-4. Synthesis of allysine ethylene acetal with PheDH/FDH[58].

Apart from L-phenylalanine, the homolog L-homophenylalanine (L-Hph), important as a component in ACE inhibitors, can be obtained from 2-keto-4-phenylbutyrate with PheDH[54]. The substrate specificity of PheDH from *Bacillus sphaericus* has been investigated by Asano et al.[55]. Table 15.3-5 compares the activities of two PheDH from *Rhodococcus rhodocrous*[8] and *Bacillus sphaericus*[55] for the transformation of aromatic and aliphatic keto acids.

Sequencing, cloning, and heterologous expression of PheDH from *Rhodococcus* was first described by Brunhuber et al.[56]. A double mutation G124A/L307V was created by site-directed mutagenesis of PheDH from *Bacillus sphaericus* to change the substrate specificity from a PheDH closer to a LeuDH. This led to a mutant with decreased activity towards L-phenylalanine and enhanced activity towards almost all aliphatic amino acid substrates, thus confirming the predictions made from molecular modeling[57].

PheDH from *Thermoactinomyces intermedius* ATCC 33205 was utilized recently to synthesize allysine ethylene acetal [(*S*)-2-amino-5-(1,3-dioxolan-2-yl)-pentanoic acid (2)] from the corresponding keto acid with regeneration of NAD^+ cofactor by FDH/formate[58] (Fig. 15.3-4); the specific activity towards the keto acid was 16% compared to the standard substrate phenylpyruvate.

The system was used in three different configurations: (i) the system with heat-dried cells from *Th. intermedius* (PheDH) and *C. boidinii* (FDH) yielded on average only 84 M% and could not be scaled up owing to lysis of the *Th. intermedius* cells; (ii) a similar system with recombinant PheDH from *E. coli* improved the yield to 91 M%; (iii) heat-dried *Pichia pastoris* containing endogeneous FDH and expressing recombinant PheDH from *Th. intermedius* yielded 98 M% with an optical purity of >98%.

Altogether, more than 200 kg of allysine ethylene acetal have been produced.

15.3.5
Summary of Substrate Specificities

The most comprehensive investigation of substrate specificity of LeuDH and PheDH has been conducted by Krix et al. (1997)[30]. Table 15.3-6 lists the relative rates of various substrates.

Table 15.3-6. Relative V_{max} values of keto acid substrates of various LeuDHs and PheDH[30].

Keto acid	*B. stearo-thermophilus* LeuDH	*B. cereus* LeuDH	*B. sphaericus* LeuDH	*Rhodococcus Rhodocrous* PheDH
Specific activity (U mg^{-1} of protein)	120	15.9	3.3	54.8
2-Oxobutyric acid	48	74	66	72
2-Oxo-3-methylbutyric acid	113	152	205	96
2-Oxo-3,3-dimethylbutyric acid	31	74	51	8
2-Oxopentanoic acid	63	81	102	157
2-Oxo-3-methylpentanoic acid	110	114	88	193
2-Oxo-4-methylpentanoic acid[a]	**= 100**	**= 100**	**= 100**	**= 100**
2-Oxo-3,3-dimethylpentanoic acid	2	11	5	4
2-Oxo-4,4-dimethylpentanoic acid	7	14	11	54
2-Oxohexanoic acid	15	63	75	250
2-Oxo-4-methylhexaoic acid	22	19	n.d.[b]	296
2-Oxo-4-ethylhexanoic acid	1	11	n.d.[b]	79
2-Oxo-4,4-dimethylhexanoic acid	0.5	1.2	0.2	146
2-Oxo-5,5-dimethylhexanoic acid	0.8	0.3	n.d.[b]	257
2-Oxo-3-cyclohexylpropanoic acid	0.8	0.1	0.3	140
2-Oxooctanoic acid	0.2	n.d.[b]	n.d.[b]	n.d.[b]
2-Oxo-3-(1-adamantyl)propanoic acid	0	n.d.[b]	n.d.[b]	16

a All V_{max} values refer to 2-oxo-4-methylpentanoic acid (= 100%), pH 8.5, T = 30 °C. Absolute activity of LeuDHs with 2-oxo-4-methyl-pentanoic acid (ketoisocaproic acid) were 120 U mg^{-1} (*B. stearothermophilus*), 15.9 U mg^{-1} (*B. cereus*) and 3.3 U mg^{-1} (*B. sphaericus*) as well as 54.8 U mg^{-1} with PheDH (*Rh. rhodocrous*).
b Not determined

LeuDHs from *B. cereus*, *B. sphaericus* and *B. stearothermophilus* display a remarkably similar substrate spectrum:

- LeuDHs accept 2-oxoacids with hydrophobic, aliphatic, branched and unbranched carbon side chains of up to six C atoms as well as some alicyclic keto acids as substrates, however, not the adamantyl group, where the geometric limit seems to be reached. 2-Oxo-3-methylpentanoic acid is the preferred substrate, the preferred chain length is C5.
- The keto acid substrate should have at least four C atoms; pyruvate is only converted at less than 3% of standard. Short-chain keto acids with branching at the C3 position are only preferred by the enzyme from *B. sphaericus*.
- The different amino acid dehydrogenases differentiate substrate side chains mainly based on steric parameters in the C3 and C4 position of branched ketoacids.
- Functionalized keto acids such as ketoglutarate are not accepted (activity <0.1% of the base case). Phenylpyruvate as a model compound of an aromatic substrate was inert[59].

A correlation of LeuDH activity with van-der-Waals volumes[60] or hydrophobicities[61] for different C atom configuration of side chains only yielded a moderate correlation[39, 61].

PheDH differs markedly from all LeuDHs, as it can convert not only aromatic substrates but also the aliphatic substrates typical for LeuDHs. Owing to the high

intrinsic specific activity of PheDH from *Rhodococcus*, in many cases the enzyme actually registers higher specific activity with many sterically demanding α-keto acid substrates than LeuDH. The substrate specifity of PheDH from *Rhodococcus rhodocrous* and *Bacillus sphaericus* seems to vary more between the two PheDHs than the specificity between the different LeuDH species. PheDH from *B. sphaericus* mainly converts (substituted) phenylpyruvates whereas the enzyme from *Rhodococcus* sp. displays a fairly high degree of activity in the presence of a phenylalkyl group in the substrate.

15.3.6
Process Technology: Cofactor Regeneration and Enzyme Membrane Reactor (EMR)

15.3.6.1
Regeneration of NAD(P)(H) Cofactors

Enzymatic reductive amination with NADH as the cofactor can only be operated on a large scale if the cofactor is regenerated. Wandrey and Kula have developed a regeneration scheme using formate as the reductant of NAD^+ generated upon reductive amination (Fig. 15.3-1). The formate is oxidized irreversibly to CO_2 by formate dehydrogenase (FDH, E.C. 1.2.1.2)[62].

For soluble reactants and products, enzymes are preferentially immobilized in an enzyme-membrane reactor (EMR). To prevent the cofactor from penetrating through the membrane, it can be enlarged with polyethyleneglycol (PEG)[63].

L-leucine was produced in an EMR with LeuDH from both *B. sphaericus*[40] and *B. stearothermophilus*[64]. LeuDH has also been employed successfully for the synthesis of L-*tert*-leucine in batch processes[39] and in its continuous version[40b, 65]. L-*tert*-leucine is an important building block for several novel pharma developments[66, 67] as well as being on intermediate for templates for asymmetric synthesis[66]. L-Phe was produced in an EMR with PheDH starting from phenylpyruvate [Fig. 15.3-5, (i)][68]. Owing to the instability and high cost of this compound, two additional processes were devised generating phenylpyruvate *in situ* (Fig. 15.3-5): (ii) intermittent oxidation of DL-phenyllactate with D- and L-hydroxyisocaproate DH (HicDH)[69], or (iii) hydrolysis of acetamidocinnamic acid (ACA) with ACA acylase[70]. For productivities of all processes, see Table 15.3-7.

Another regeneration scheme for NADH from NAD^+ utilizes glucose which is oxidized to gluconic acid with the help of glucose dehydrogenase (see Fig. 15.3-3 for an example)[51]. Regeneration to NADPH from $NADP^+$ can be afforded by glucose-6-phosphate dehydrogenase with glucose-6-phosphate as the substrate[71, 72]; the system, however, has not found widespread use yet, probably owing to the higher price of $NADP^+$ vs. NAD^+ and the cost associated with the generation of glucose-6-phosphate from glucose.

With the advantage of the potentially quantitative use of a keto acid substrate and with suitable processes of cofactor regeneration, reductive amination of keto acids is an interesting route to α-amino acids worthy of consideration in comparison with more established routes.

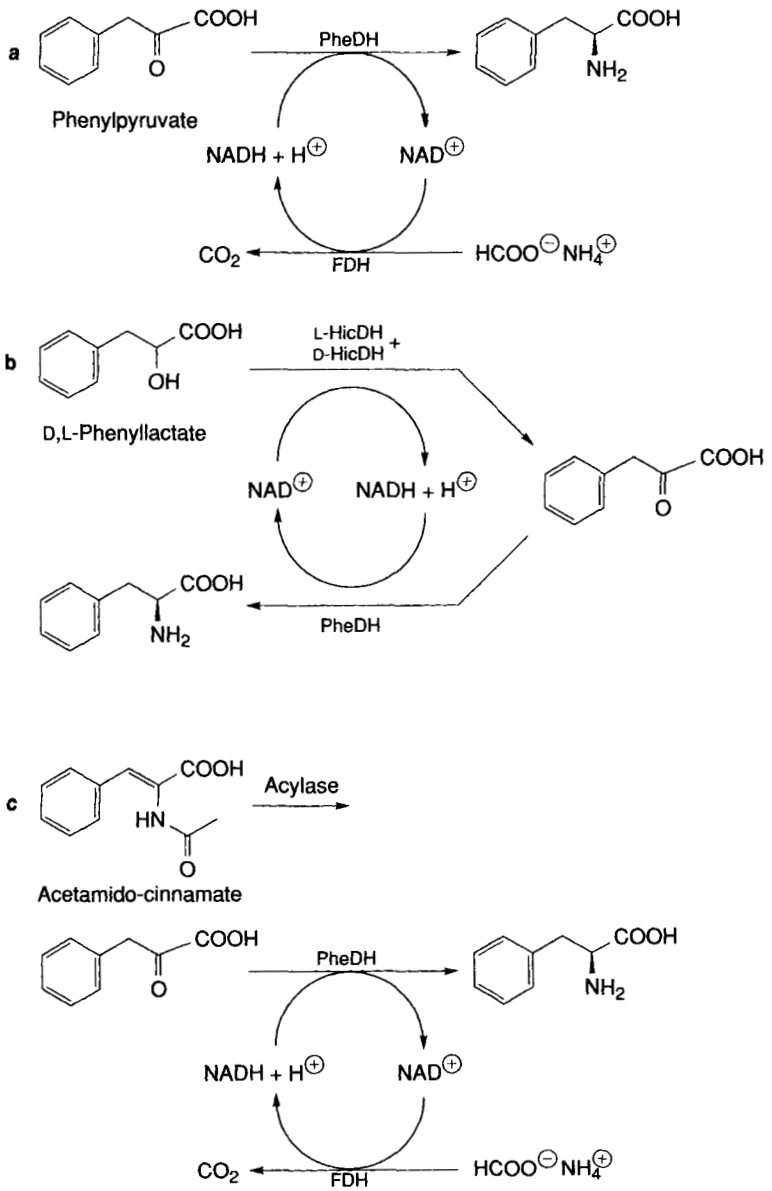


Figure 15.3-5. Enzymatic routes to L-phenylalanine via phenylpyruvate[9]. (i) Reductive amination of phenylpyruvate by PheDH with simultaneous NADH regeneration using FDH.
(ii) Oxidation of DL-phenyllactate with D- and L-2-hydroxy-4-methylpentanoate (HicDH) and simultaneous reductive amination of the phenylpyruvate formed *in situ* with PheDH. NADH is "substrate-coupled" regenerated from phenyllactate. (iii) *In situ* formation of phenylpyruvate by enzymatic deacetylation of *N*-acetamidoocinnamic acid by the respective acylase followed by simultaneous reductive amination with PheDH.

Table 15.3-7. Continuous production of L-amino acids with the aid of dehydrogenases in an enzyme membrane reactor[9].

AADH	Regeneration enzyme(s)	Precursor	Product	Product conc. (mM)	Degree of conversion	s.t.y. g/(Lxd)	Enzyme consumptions (U kg^{-1})	Ref.
LeuDH	FDH	oxomethyl-pentanoate	L-leu	80	80	250	300/300 (LeuDH,FDH)	40c
LeuDH	D-HmpDH L-HmpDH	DL-OH-methyl-pentanoate	L-leu	70	70	72	730/350/650 (LeuDH, D-HmpDH, L-HmpDH)	40a
LeuDH	D-HmpDH L-HmpDH	D,L-OH-methionine	L-met	240	60	143		40c
LeuDH	FDH	trimethyl-pyruvate	L-tle	425	85	640	1000/2000 (LeuDH, FDH)	40c
AlaDH	D-LDH L-LDH	D,L-lactate	L-ala	184	46	134	4700/2600 (LeuDH, FDH)	40a
PheDH	FDH	phenyl-pyruvate	L-phe	114	95	456	1500/150 (PheDH, FDH)	68
PheDH	D-HmpDH L-HmpDH	D,L-phenyl-lactate	L-phe	22	43	28		69
PheDH + ACA acylase	FDH	acetamido-cinnamate	L-phe	70	88	277	1170/1770/400 (acylase, PheDH, FDH)	70

HmpDH: 2-hydroxy-4-methylpentanoate-DH; LDH: lactate dehydrogenase; L-tle: L-tert-leucine

15.3.6.2
Summary of Processing to Amino Acids

The production of L-*tert* leucine on a multi-100 kg scale and of L-neopentylglycine on a 30 kg scale with LeuDH from *B. stearothermophilus* demonstrates the suitability of enzymatic reductive amination on a large scale and even for slow substrates. The economics of the process is influenced decisively by the retention and regeneration of both production (AADH) and regeneration enzyme (FDH). If yields of less than 100% are acceptable enzyme consumption can be lowered by running the process in a continuous mode[40b]. Owing to the broad substrate specificity of AADHs, reductive amination can be utilized especially for the synthesis of hydrophobic amino acids. LeuDH and PheDH feature complementary specificities for aliphatic and aromatic L-amino acids. Both enzymes are enantioselective to the highest degree and stable in a coupled process with FDH. As non-enzymatic processes of reductive amination often lead to low yields and enantioselectivities[73–76], enzymatic schemes are superior to chemical ones. Additionally, enzymatic reductive aminations are conducted solely in water so that organic solvents can be avoided.

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16
Oxidation Reactions

16.1
Oxygenation of C-H and C=C Bonds

Sabine Flitsch, Gideon Grogan and D. Ashcroft

16.1.1
Introduction

Reactions catalyzed by oxygenase enzymes (mono or dioxygenases) are interesting for applications in organic synthesis. There are numerous examples of such reactions in biological systems, yet there are few chemical reagents or catalysts that can compete with biocatalysts. Examples of monoxygenases catalyzed biotransformations are shown in Figure 16.1-1. These include heteroatom oxygenation, aromatic hydroxylation, Bayer-Villiger oxidation, double-bond epoxidation and hydroxylation of non-activated hydrocarbon atoms. The latter can occur with regio-, stereo-,

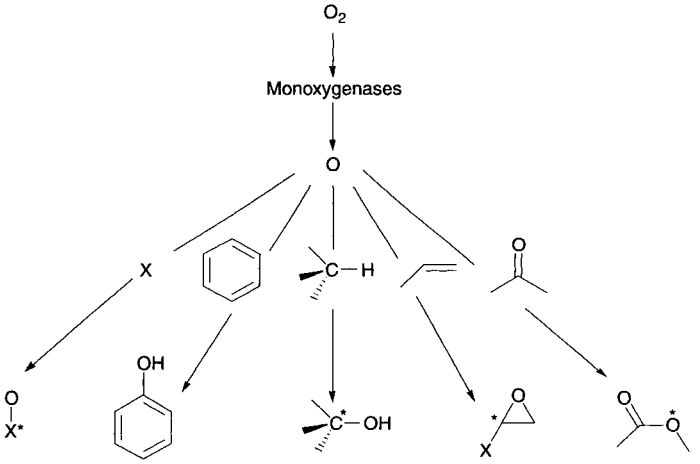


Figure 16.1-1. Some examples of monooxygenase-catalyzed biotransformations.

and in some cases enantioselectivity that is difficult to achieve using conventional chemistry. There is a large body of literature describing the exploitation of these oxygenase enzymes for synthetic applications, and the current chapter will only give a few representative examples of what has been done.

There are few reports on biotransformations using isolated oxygenase enzymes because of several problems with cell free enzymes. Many of the oxygenases are membrane-bound, and require a complex set of co-factors and co-proteins. Despite the fact that a large number of their genes have been identified, the enzymes themselves are difficult to isolate and quite unstable. Thus, oxidative bioconversions, especially on an industrial scale, generally use whole-cell bioconversion techniques, which makes them less accessible for use by organic chemists in organic synthesis laboratories. However, very recently new techniques have been developed for the isolation, cloning and over-expression of oxygenases in heterologous expression systems, and the number of reports using isolated systems is increasing. These new developments will be discussed at the end of the chapter, and point to the possibility of overcoming technical difficulties that have hampered the application of oxygenase systems in biocatalysis in the past.

A number of excellent reviews with comprehensive coverage on the literature of biooxidations have appeared in journals and books[1–8]. In this chapter we will only try to highlight some of these biotransformation reactions, in particular hydroxylation of non-activated carbon atoms and double-bond epoxidation reactions.

16.1.2
Hydroxylating Enzymes

Hydroxylation reactions in nature are generally catalyzed by monooxygenase, a subclass of the oxidoreductase enzyme group. These enzymes are very important and ubiquitous proteins found in almost all living cells, ranging from bacterial to mammalian. One of the most important groups of this type of enzyme is the cytochrome P450 family. These are heme-dependent monooxygenases whose essential role, among other functions, is to ensure detoxification of exogenous compounds by rendering these very often lipophilic molecules water soluble, thus facilitating their excretion. Because of this essential function, mammalian monooxygenases have been thoroughly studied in the context of drug metabolism[1, 9–14].

The majority of the cytochrome P450 systems reported to date are multicomponent, requiring the involvement of additional proteins for transport of reducing equivalents from NAD(P)H to the terminal cytochrome P450 component. Increasing attention is given to microbial monooxygenases, in particular in their application for biotransformations. One of the earliest and most important industrial applications of microbiologically mediated bioconversions is the 11-α-hydroxylation of progesterone using *Rhizopus arrhizus* cells (Figure 16.1-2)[15]. Microbial monooxygenases tend to be soluble enzymes that can be purified fom cell free extracts, and a number of crystal structures of microbial monooxygenases are now available[16, 17]. The first X-ray structure for P450 was that of cytochrome $P450_{cam}$, which was isolated from *Pseudomonas putida*, and catalyzes the 5-*exo* hydroxylation of its natural substrate D-camphor to 5-*exo* hydroxycamphor as shown in Figure 16.1-2.

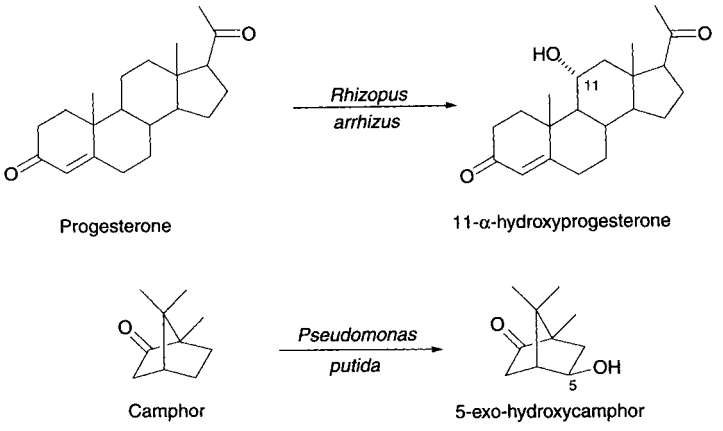


Figure 16.1-2. Regio- and stereoselective microbiological hydroxylation of progesterone and D-camphor.

The $P450_{cam}$ enzyme has served as a model system for general studies of cytochrome P450 enzymes in terms of structure, function and mechanism[16, 18]. The exquisite regio- and stereoselectivity can be explained by the active site geometry of the $P450_{cam}$, which shows several van der Waals interactions with hydrophobic side chains and a key hydrogen bond between tyrosine 96 and the carbonyl oxygen of the substrate. Removal of the tyrosine-96 hydroxyl group by site-directed mutagenesis or removal of the carbonyl oxygen by using camphane results in loss of selectivity[19].

There are also an increasing number of non-P450 type biohydroxylases. Examples are the *n*-octane ω-hydroxylase of *Pseudomonas oleovorans* and the *n*-decane hydroxylase of *Pseudomonas denitrificans*, which have been shown to be also responsible for epoxidation of 1-octene and for *O*-demethylation of heptyl methyl ether[20–24].

Similarly, the progesterone 9-α-hydroxlase from *Norcardia* sp., one of the first microbial hydroxlases obtained in crude form, was shown not to be a cytochrome P450 protein[25]. Interestingly, this enzyme allows for the functionalization of the steroid skeleton, thus opening the way to production of the C-11-oxygenated corticosteroids[26] as shown in Figure 16.1-3.

Other very important examples of non-cytochrome P450 enzymes are methane monooxygenases. These appear to be more reactive than P450 oxygenases and are able to catalyze the conversion of methane to methanol, chemically one of the most difficult steps[27]. These enzymes have been shown to reductively activate dioxygen for incorporation into a wide variety of hydrocarbon substrates, including alkanes, alkenes and alicyclic or aromatic hydrocarbons. The enzyme harbors a hydroxy-bridged dinuclear iron cluster in its active site, and its structure has been determined by X-ray crystallography[28].

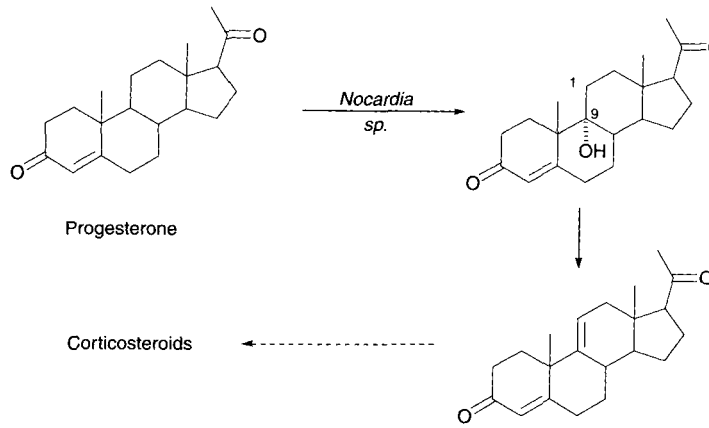


Figure 16.1-3. Use of 9-α-hydroxylation of progesterone as a way to corticosteroids.

16.1.2
Hydroxylating Enzymes

The active site of P450 monooxygenases contains an iron-heme center that is directly involved in the oxidation process by activating molecular oxygen. The catalytic cycle by which cytochrome P450-mediated alkane hydroxylation occurs is by now well studied[1, 18]. The reaction cycle of cytochrome $P450_{cam}$ is outlined in Figure 16.1-4. This mechanism involves (i) reversible substrate binding which converts the six-coordinate, low spin form of the protein to the penta-coordinate high-spin form, (ii) electron reduction of the ferric substrate-enzyme complex by flavoprotein NADPH-

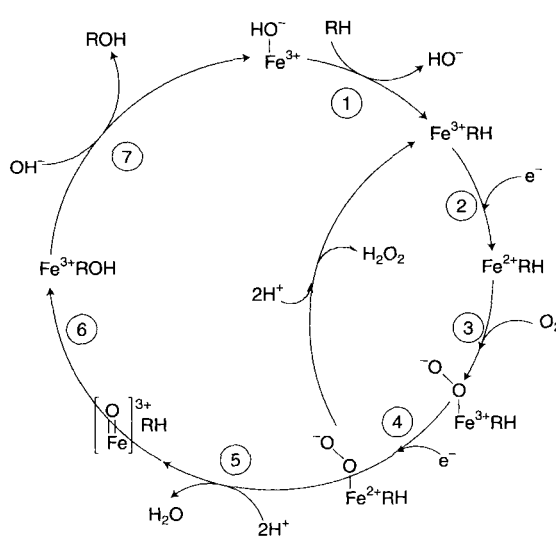


Figure 16.1-4. The catalytic cycle of cytochrome P450 enzymes.

cytochrome P450 reductase leading to the ferrous enzyme, (iii) binding of molecular oxygen to give the six-coordinate iron-dioxygen intermediate, (iv) + (v) reduction of this species with a second electron and addition of two protons, thus leading to an activated oxygen intermediate, (vi) insertion of an oxygen atom into the substrate, and (vii) release of the iron atom in its original ferrous state. Apart from the products of steps (iv) and (v), all these intermediates have now been investigated by crystallography using trapping and cryocrystallography methods [18].

The exact mechanistic details of oxygen insertion into the C-H bond are still the subject of intense discussion. One of the most popular proposals appears to be that of the so-called "rebound mechanism", which proceeds by an initial hydrogen abstraction from the alkane (RH) by the active oxygen intermediate to form a radical R and a hydroxo-iron species as intermediates. The radical then rebounds on the hydroxy group and generates the enzyme-product species [29]. Alternative proposals involve cationic intermediates [30] or two-state reactivity with multiple electromer species for epoxidations [31].

It should be noted that the same cytochrome P450 enzyme is able to achieve reactions as different as double-bond epoxidation or heteroatom demethylation. Thus it appears clear that the chemo-, regio- and stereoselectivity of the reaction is a function of the nature and the fit of the substrate, or, more properly, of its transition state with the protein, rather than being governed by enzyme reaction specificity.

It is obvious that all these very powerful enzymes are of high synthetic value for the organic chemist since they prove to be able to achieve, at normal temperature and in aqueous media, reactions which are very difficult, if not impossible, to perform using conventional chemistry. One additional bonus offered by these biological tools is their generally high selectivity. It is thus understandable that a variety of oxygenative biotransformations have been explored using numerous substrates. We will focus in the following pages on two such particularly interesting reaction types, namely the hydroxylation of non-activated carbon atoms and the stereospecific epoxidation of "*isolated*" double bonds.

16.1.4
Hydroxylation of Non-Activated Carbon Atoms

16.1.4.1
Hydroxylation of Monoterpenes

Because of their involvement in the flavor and fragrance industry, monoterpenes are one type of natural compounds which have been considered as interesting substrates for biohydroxylation studies. For instance, geraniol, nerol and linalool were studied by different groups and were shown to lead to the 8-hydroxylated products with the fungus *Aspergillus niger* [32] as well as with four strains of *Botrytis cinerea* [33]. Interestingly, the same 8-hydroxylated products were found starting from the corresponding acetates, which were shown to be hydrolyzed to the starting alcohol by the fungus *Aspergillus niger* prior to hydroxylation [34]. This C-8 regioselectivity has also been observed in hydroxylations of geraniol and nerol with reconstituted

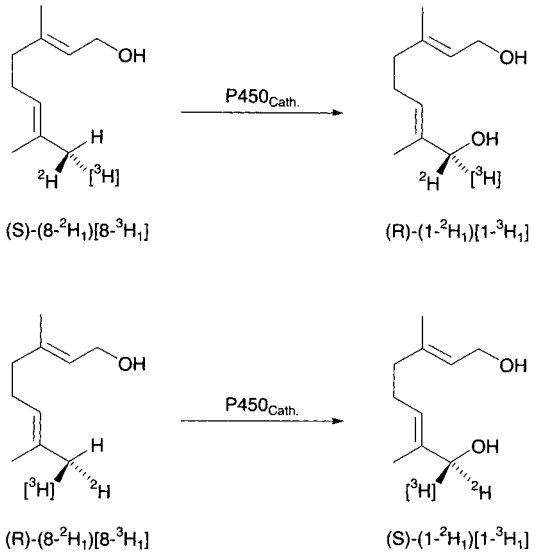


Figure 16.1-5. Retention of configuration during the hydroxylation at C-8 of isotopically labeled geraniol.

hydroxylating enzyme systems from rabbit liver[35–39] and from plant cells like *Vinca rosea* as well as *Catharanthus roseus* (L.) G. DON[40].

In this last case, incubation of different ^{13}C- and ^{2}H-labeled geraniols revealed that hydrogen abstraction is completely regioselective in favor of the CH_3 group *trans* to the chain at C-6, i.e. at position C-8. An intramolecular isotope effect of K_H/K_D 8.0 was determined, suggesting that the hydrogen abstraction is one of the major rate determining steps. Furthermore, Fretz and Woggon[41] have studied incubation of the (*R*)(8-$^{2}H_1$) (8-$^{3}H_1$) and (*S*)(8-$^{2}H_1$) (8-$^{3}H_1$) geraniols (Fig. 16.1-5). This resulted in the formation of the chiral 8-hydroxy products, thus indicating clearly retention of configuration during the allylic hydroxylation process. Interestingly, in neither of these cases has an allylic radical rearrangement (migration of the double bond) been observed.

Bioconversions of (+)-limonene, the major constituent of citrus essential oils, have also been studied in recent years in order to afford biotechnological routes to interesting products of natural source valuable for the perfume and/or flavor industries. For instance, (+)-limonene was shown to be transformed by *Pseudomonas gladioli* to (+)-α-terpineol (one of the most commonly used products in fragrances and flavors i.e. lemon, nutmeg, orange, ginger, peach and spices), which is resistant to further degradation by the bacterium, and to (+)-perillic acid, which is further metabolized[42]. The corresponding levorotatory limonene antipode is known as being the primary olefinic constituent of the volatile oils of immature *Mentha piperita* (peppermint), *Mentha spicata* (spearmint) and *Perrilla frutescens* leaves, whereas (-)-menthone, (-)-carvone and (-)-perillyl aldehyde, respectively, are the major oxygenated compounds. The enzymatic hydroxylation of (-)-limonene at C-3, C-6 and C-7 to the corresponding derivatives has been studied using light membrane preparations from leaves of each of these plants. It has thus been shown that they lead to the

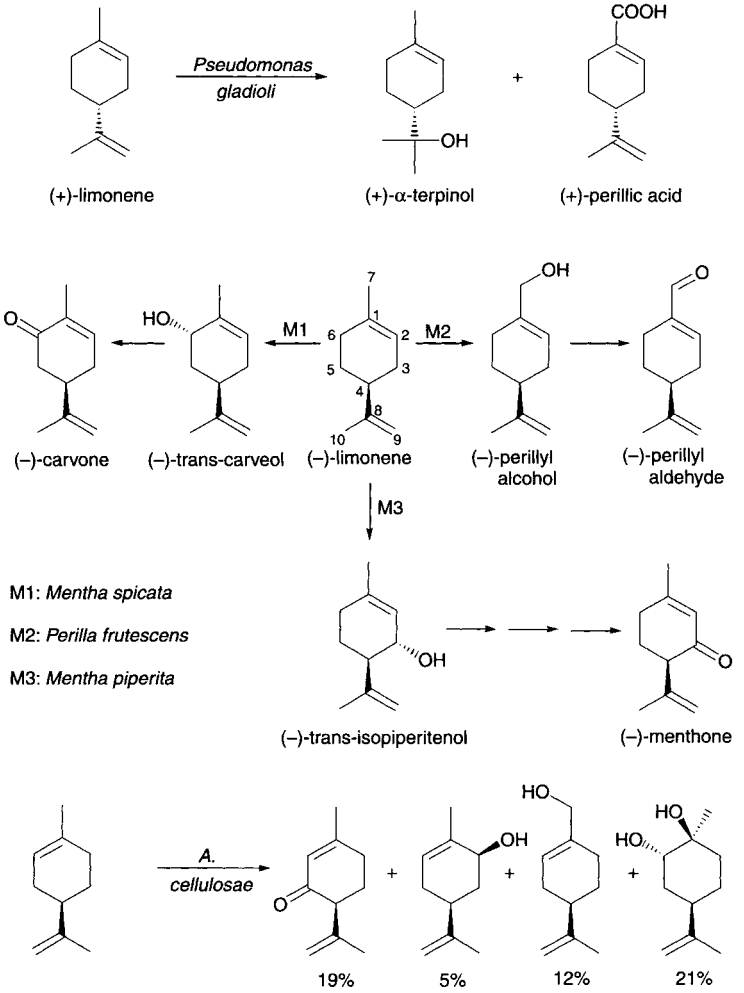


Figure 16.1-6. Bioconversion of limonene using various biocatalysts.

corresponding oxygenated compounds in a mutually exclusive manner in each species. This suggests very strongly that different forms of cytochrome P450 are present in each type of plant, each one showing an exclusive regiochemistry of oxygen insertion[43] as shown in Fig. 16.1-6.

Another terpene interesting for flavor chemistry is β-ionone (Fig. 16.1-7). Its microbiologically mediated transformation has been explored in order to afford a mixture of derivatives that is utilized as an essential oil of tobacco, used for tobacco flavoring at the ppm level[44, 45]. One of the best microorganisms capable of converting β-ionone to the desired mixture of its useful derivatives was an *Aspergillus niger* strain. This process has been recently improved using bioconversion in the presence of organic solvents and immobilization techniques. Thus, this fungus

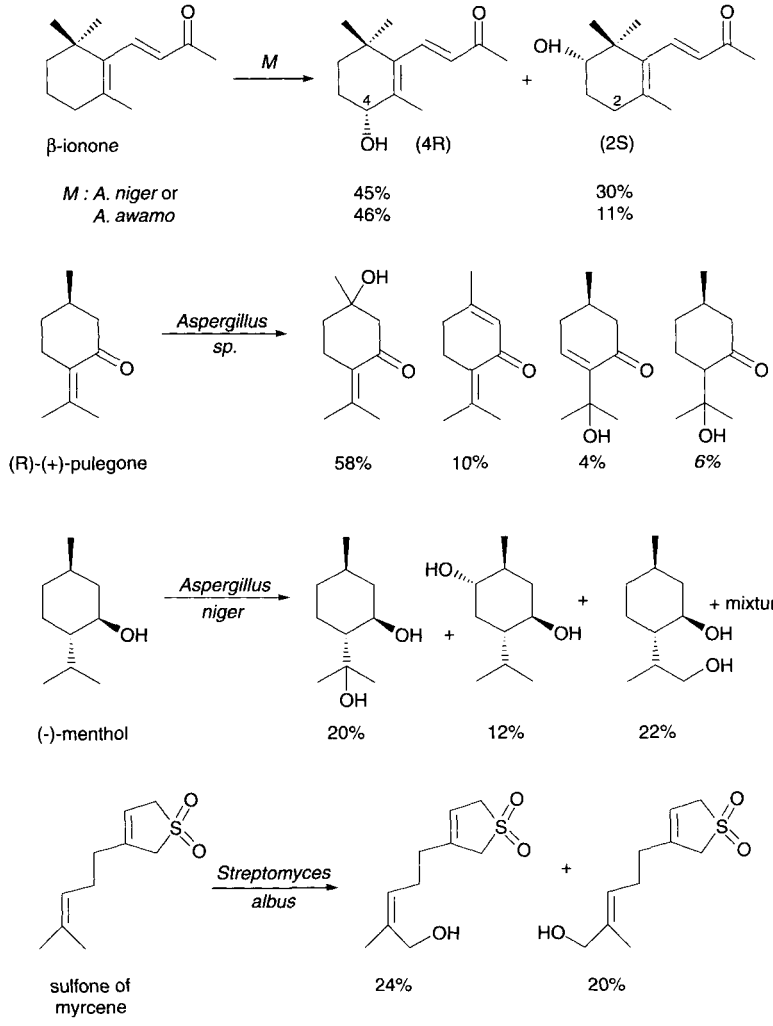


Figure 16.1-7. Biohydroxylations of various monocyclic terpenes.

could be repeatedly used for microbial conversion of β-ionone in the presence of isooctane for more than 480 h[46]. Another *Aspergillus* strain, *A. awamori*, has also been shown recently to achieve hydroxylation of β-ionone. This led to a mixture of two alcohols, the major product being a building block usable for further synthesis of abscisic acid (an important phytohormone) analogs[47].

Other monocyclic terpenes like for instance R-(+)-pulegone (a mint-like odor monoterpene ketone which constitutes the main component of *Mentha pulegium* essential oil)[48] menthols, terpinolene and carvotanacetone[49] have been investigated. All these substrates proved to be transformed by various *Aspergillus* strains, including *A. niger*, leading essentially to monohydroxylation. Also, monocyclic

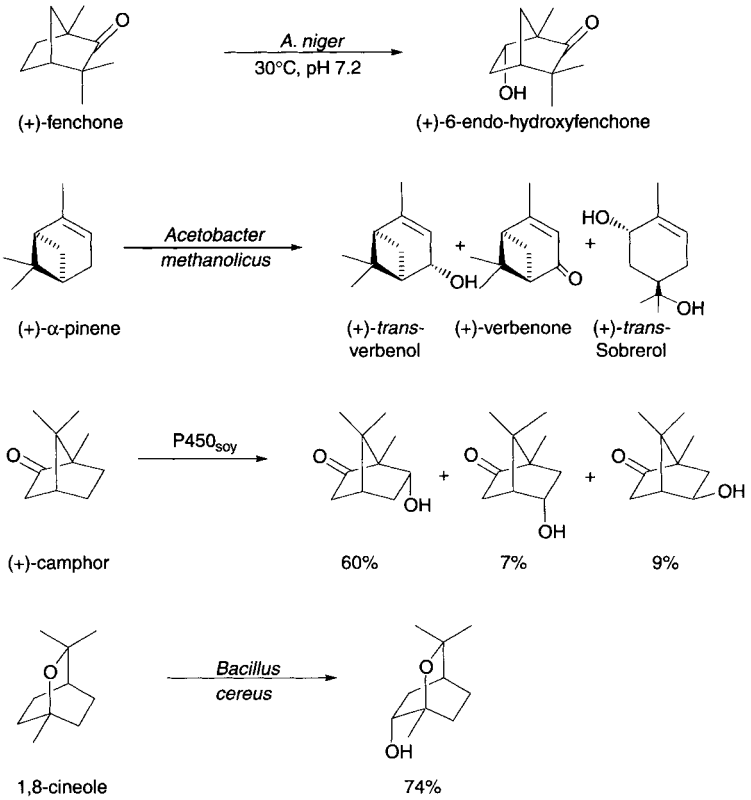


Figure 16.1-8. Biohydroxylation of various bridged bicyclic terpenes.

sulfoxide derivatives of the linear terpenes myrcene and ocimene were shown to be good substrates for several bacteria and fungi, whereas they were themselves only very poorly transformed[50].

Finally, some bicyclic monoterpenes have also been recently described to be subject to microbiological hydroxylation (Fig. 16.1-8). For instance (+)-fenchone was transformed to (+)-6-*endo*-hydroxyfenchone by *A. niger*[51], and α-pinene led to the predominant metabolites (+)-trans-verbenol, (+)-verbenone and (+)-trans-sobrerol by the action of several strains of the methylotrophic species *Acetobacter methanolicus*[52]. (+)-Camphor is transformed to the major metabolite 6-*endo*-hydroxy-camphor by cytochrome $P450_{soy}$ enriched intact cells of *Streptomyces griseus*[53], and 1,8-cineole (the major component of the oil from leaves of *Eucalyptus radiata* var.) is hydroxylated to 6-(*R*)-*exo*-hydroxy-1,8-cineole by the bacterium *Bacillus cereus* following a high yielding (74%) and stereospecific route[54]. Interestingly, this same bacterium had been previously described to be able to achieve hydroxylation of 1,4-cineole yielding good yields of essentially pure 2-(*R*)-*endo* and 2-(*R*)-*exo*-hydroxy-1,4-cineole[55].

16.1.4.1
Hydroxylation of Monoterpenes

Similarly to monoterpenes, several studies aimed at the microbiological hydroxylation of sesquiterpenes have been described, and a review has summarized the most interesting results obtained in this area[56]. Since then, additional examples have been published. Thus, three germacrone-type sesquiterpenoids, (+)-germacrone-4,5-epoxide, germacrone and (+)-curdione, were described as being transformed by *Aspergillus niger*[57]. The interesting feature of these results is the fact that they essentially led to hydroxylated guaiane-type sesquiterpenoids (together with allylic alcohols and spirolactone) which arise from transannular cyclization of the carbon

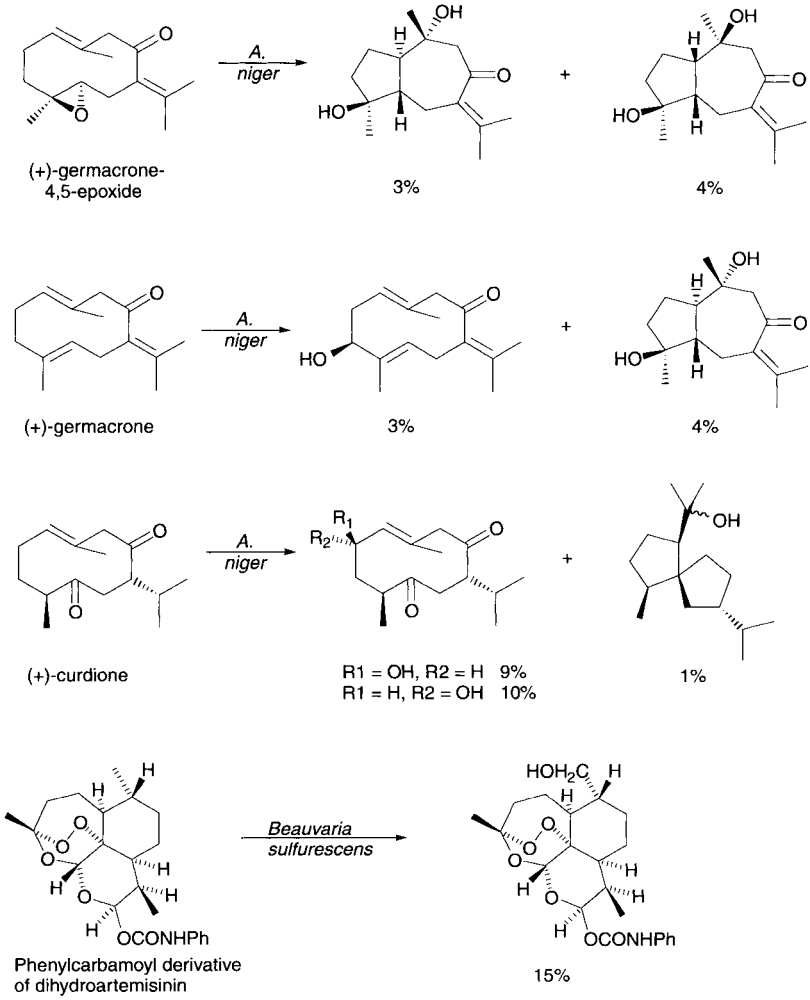


Figure 16.1-9. Biohydroxylation of various higher terpenes.

skeleton as shown in Fig. 16.1-9. The biohydroxylation of an *N*-phenylcarbamoyl derivative of dihydroartemisinine by the fungus *Beauveria sulfurescens* has been described[58]. This allowed the preparation of new and novel derivatives of artemisinine, a drug known to be active against *Plasmodium falciparum*, the strain responsible for malaria, which claims more than one million lives a year. The C-10-*N*-phenylcarbamoyl derivative of dihydroartemisinine, a highly oxygenated sesquiterpene, is thus converted into its 14-hydroxymethyl derivative in 15% yield. Although this yield can be considered as rather modest, this biohydroxylation is interesting since it allows us to prepare derivatives which retain the peroxide group required for biological activity of these drugs. Also, it emphasizes the possibility to favor biohydroxylation by introducing an amide or urethane group into a substrate, as already observed on other model substrates (Fig. 16.1-9)[59, 60].

A series of biotransformations of 6β-santonin and of some of its derivatives, achieved by *Curvularia lunata* and *Rhizopus nigricans* cultures, have also been described (Fig. 16.1-10)[61]. Depending on the strain used and on the starting substrate, several metabolites were obtained, including products resulting from hydroxylation as well as double bond and/or carbonyl reduction. The same group also described biotransformations of several 1,6-difunctionalized eudesmanes leading to 12-hydroxy derivatives which are interesting intermediates for the synthesis of 6,12-eudesmanolides[62]. Similarly, starting from 6β-acetoxyeudesmanone, biohy-

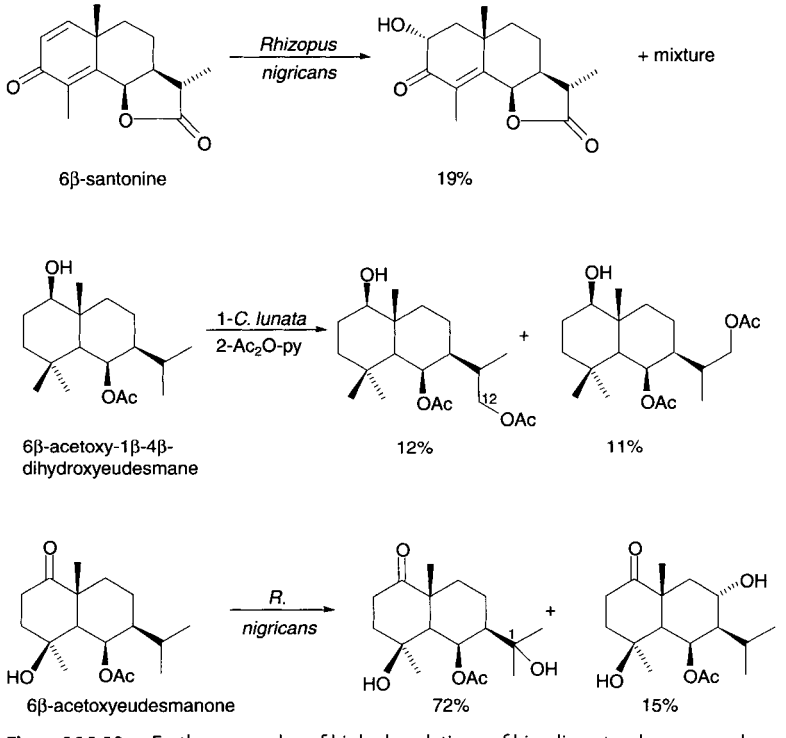


Figure 16.1-10. Further examples of biohydroxylations of bicyclic natural compounds.

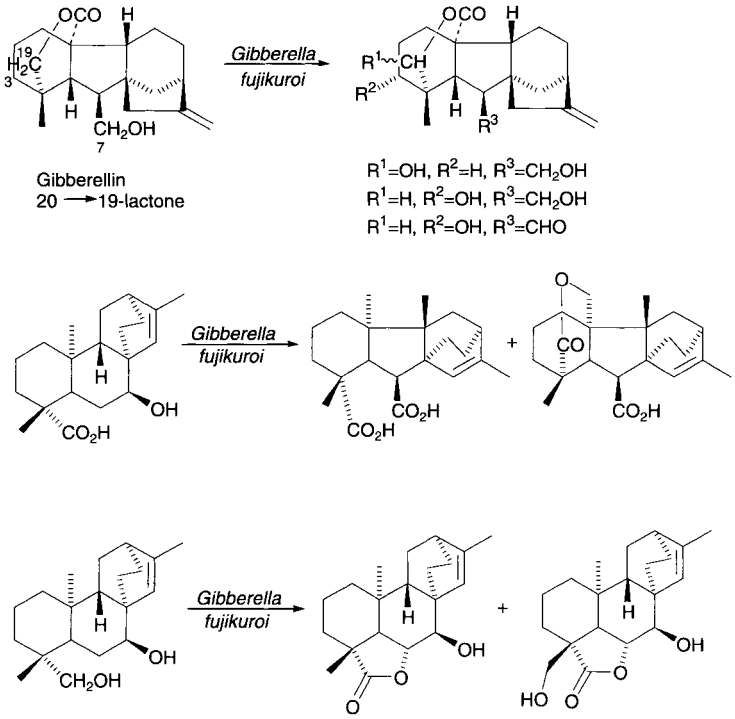


Figure 16.1-11. Some examples of diterpenes hydroxylation by *Gibberella fujikuroi.*

droxylation was achieved at C-11 by the fungus *Rhizopus nigricans,* thus opening another way to the synthesis of the same lactone targets[63].

Several diterpenes have also been described recently to be subject to microbiological hydroxylations. Thus, as shown in Fig. 16.1-11, a compound prepared from gibberellin A_{13} was transformed by the fungus *Gibberella fujikuroi,* affording three metabolites, two of these arising from hydroxylation at its 3α- and 19-positions[64]. Similarly, the same fungus was shown to transform isoatisene derivatives into rearranged isoatisagibberellin derivatives[65].

Sclareol, a natural product first isolated from the essential oil of *Salvia sclarea* L. (Labiatae) in 1931, is used for diverse applications in the perfumery and flavoring industries and in folk medicine. This diterpene has been described recently to be hydroxylated by three strains, i.e. *Cunninghamella* sp., *Septomyxa affinis*[66] and *Mucor plumbeus*[67, 68], leading essentially to hydroxylation reactions on the A ring of this compound (Fig. 16.1-12). Some of these metabolites could be used for further synthesis of some biologically active targets or as mammalian metabolism models.

Amazingly, as in the case of sciareol, the presence of an oxygenated function on the C ring position of grindelic acid again orients the hydroxylation process towards the A ring, since mainly 3β-hydroxylation is observed[69]. This is to be compared with the results obtained in studying the behavior of *Rhizopus* and *Aspergillus* strains as potential hydroxylating species for kaurene sesquiterpenes[70] (Fig. 16.1-13). Inter-

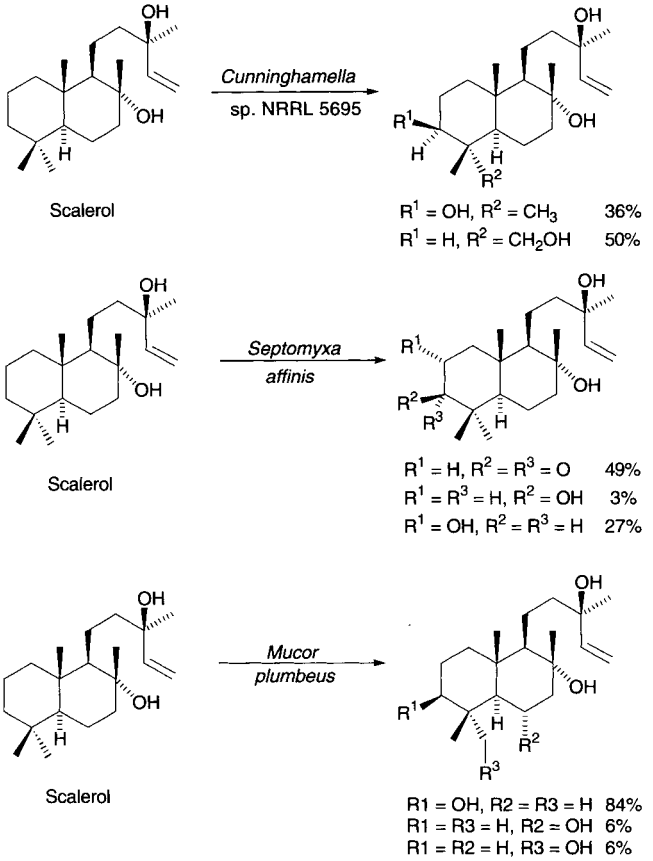


Figure 16.1-12. Hydroxylation of natural sclareol using different microorganisms.

estingly, the fact that the starting substrate bears an oxygenated function on the A ring now orients the oxidations catalysed by *R. nigricans* toward the C ring (and in particular to the C-13 position). This nicely resembles the results previously observed in the steroid family by Jones and coworkers [71], and once more emphasizes the role of a preexisting oxygenated function which operates as an anchoring and thus a site-directing entity inside the hydroxylating active site.

By modifying the location of this function on the starting material, it should therefore be possible to orient the hydroxylation locus differently. This is nicely exemplified by hydroxylation of stemodine, a diterpene bearing a preexistent OH group at position 2 of the A ring. Hydroxylation by *C. elegans* and by *Polyangium cellulosum* then orients the hydroxylation process toward positions 17 and 19 [72].

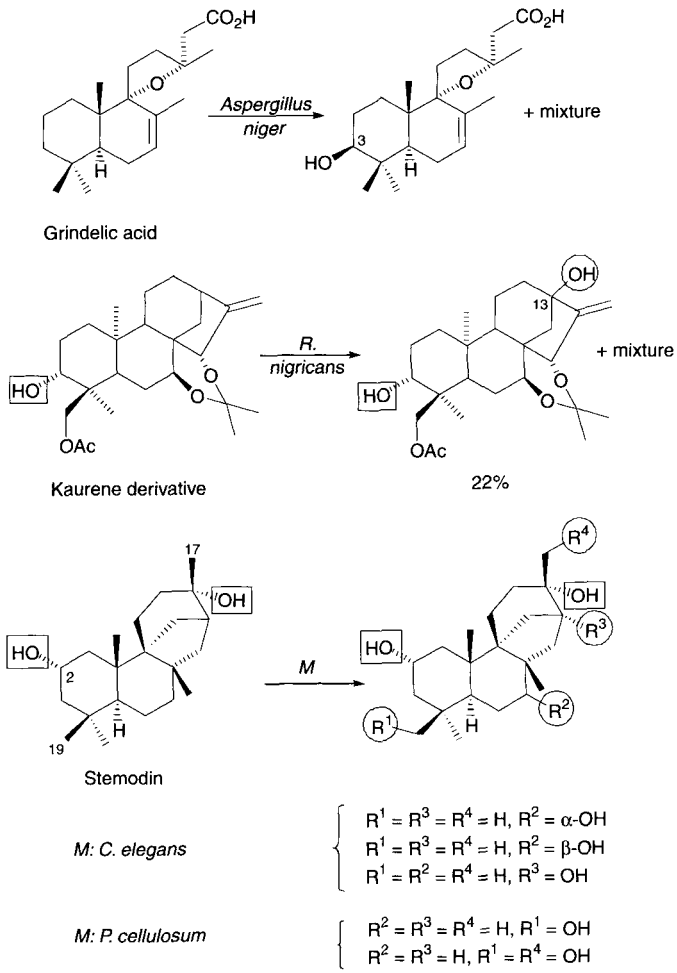


Figure 16.1-13. Effect of a preexisting oxygenated function on the orientation of a biohydroxylation process.

16.1.4.3
Hydroxylation of Steroids

Because of their utmost importance as bioactive molecules, steroids have been the most thoroughly studied family as far as microbiological hydroxylations are concerned.

The most important features and references have been put together by Holland in his important monograph[73]. At the present time, one could presumably almost consider that one or even several strains are known which are able to introduce a hydroxyl group at every carbon atom of the steroidal framework. Obviously, however, further work will have to be achieved in order to improve the selectivities and yields

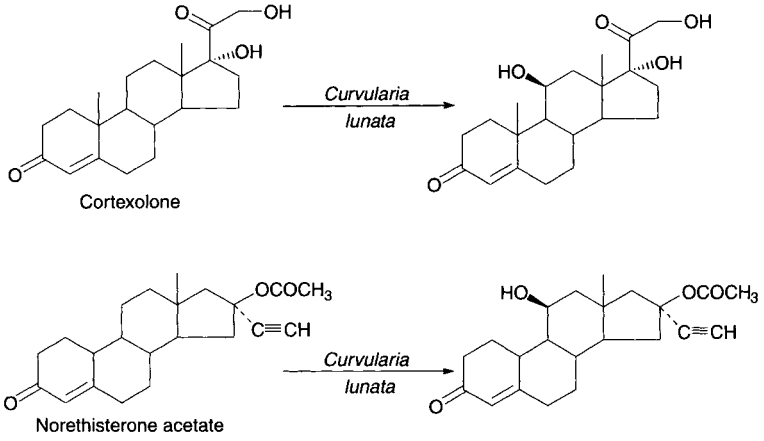


Figure 16.1-14. Examples of steroid hydroxylations by *Curvularia lunata*.

of these bioconversions. Thus, the course of the 11β-hydroxylation of cortexolone by *Curvularia lunata*, a hydroxylation of considerable commercial importance, has been more recently reexamined. The work described by Chen and Wey[74] focused on the improvement of this process by studying the characteristics of mycelial growth as well as the role of substrate addition time and dissolved oxygen tension. These studies have provided some more insight into the fundamental aspects of this biotransformation. The 11β-hydroxylation of norethisterone acetate by the same microbial strain has also been described[75] (Fig. 16.1-14).

16.1.4.4
Miscellaneous Compounds

Although being initially the major part of literature concerning microbiological hydroxylations, natural compounds of the terpene or steroid families have not been the only ones to be studied in this context. Indeed, more recently studies have focused on using biohydroxylations to provide interesting synthetic intermediates, in particular chiral intermediates.

Linear or branched-chain alkanes have been shown previously to undergo hydroxylation by various microorganisms, and can lead for instance to fatty acids, hydroxyacids or α-dicarboxylic acids of commercial importance. *Pseudomonas oleovorans* is one of the strains capable of achieving such transformations, but this process suffers from the fact that the monocarboxylic acids formed in the first step are submitted to β-oxidation and thus used as a source of energy and carbon. Such problems can now be overcome using heterologous expression of P450 genes in microorganism, which will be discussed later on.

An interesting aspect of biohydroxylations is that the biocatalyst can in principle generate one single enantiomer starting from a prochiral substrate – a reaction which could be defined as *"enantiogenic"*[76]. This enantiogenic hydroxylation can lead to enantiopure compounds by stereospecific attack of one single enantiotopic

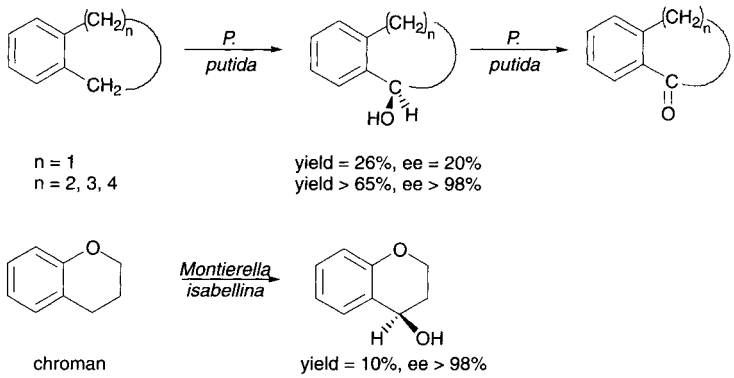


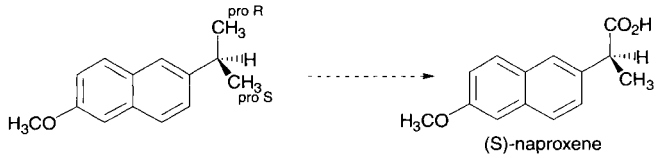
Figure 16.1-15. Examples of regio- and stereoselective benzylic hydroxylation.

face of the starting substrate. Biohydroxylation reaction can therefore be used to prepare high value chiral synthetic intermediates from low value prochiral starting materials, and there are now a number of reports of such reactions.

Thus, benzocycloalkenes have been described to undergo bacterial hydroxylation by the *Pseudomonas putida* strain UV4. As shown in Fig. 16.1-15, this yielded exclusively hydroxylation at the benzylic position, and also one single enantiomer, i.e. the (*R*)-alcohol. The biotransformation of benzocyclobutene proved, however, to be different from that observed for higher benzocycloalkenes, presumably because of its particular chemical reactivity[77]. A similar result has been observed by Holland and coworkers in the course of chroman biotransformation by the fungus *Mortierella isabellina*[78], which leads, although in low yield, to the benzylic (*R*)-alcohol.

Another interesting example of asymmetric synthesis from a prochiral substrate is the preparation of (*S*)-naproxen, a non-steroidal anti-inflammatory drug. It has been shown that several strains are able to regioselectively oxidize one of the enantiotopic methyl groups of the isopropyl moiety. This allows the preparation of the corresponding acid, which is obtained with high enantiomeric purity (Fig. 16.1-16).

Next to *Aspergillus niger* the fungus *Beauveria bassiana* (previously classified as *Sporotrichum sulfurescens* and *B. sulfurescens*) is one of the most frequently used fungal biocatalyst[2]. In particular, hydroxylations of piperidine and pyrrolidine derivatives have been studied by several groups, and interesting regio- and ster-



Organism	ee (%)
Cordyceps militaris	99
Graphinium fructicola	96
Exophiala mansonni	68
Exophiala jeanselmei	66

Figure 16.1-16. Selective hydroxylation of one enantiotopic methyl group as an approach to optically pure Naproxene.

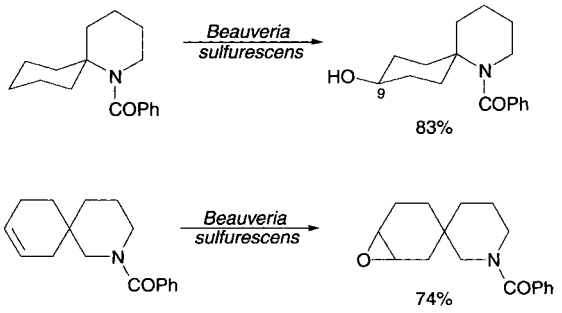


Figure 16.1-17. Hydroxylation versus epoxidation of two spiro-bicyclic amides.

eoselectivities have been reported[79–83]. It should be noted that the ring nitrogen generally needs to be protected for a successful biohydroxylation. This can be used to advantage since the choice of protecting group can influence the regio- and stereochemistry of the hydroxylation[84]. Some examples of hydroxylations of spirobicyclic amides are shown in Fig. 16.1-17. Similarly to previous results described by Fonken and coworkers[85, 86] and by Furstoss and coworkers[87, 88], these led to good yields of hydroxylated products. In all these cases, the regioselectivity of the reaction is partly or even exclusively oriented toward the C-9 carbon atom, a result which could have been predicted on the basis of the previously described results. Interestingly, a similar substrate bearing a double bond at carbon C-9 led to the corresponding epoxide.

Because they constitute partial structures of various higher terpenes and/or steroids, enantiomers of different substituted hexahydronaphthalenones are pivotal intermediates in the total synthesis of these target compounds. Therefore, several differently substituted octalone derivatives have been studied for microbiological hydroxylations. These substrates were prepared in optically active form by chemical synthesis from (*S*)-(+)-Wieland-Miescher's ketone and were submitted for screening with nine strains known to hydroxylate polyterpenic or steroidal substrates. Thus, submitted to a culture of *Rhizopus arrhizus*, these substrates led to allylic hydroxylation at the B ring, as shown in Fig. 16.1-18[89, 90]. Similar results were obtained by Azerad and coworkers[91] in the same series, starting from differently substituted octalones. These authors have investigated the biotransformation of their substrates with a variety of fungal strains. For most of these strains, the (*R*)-enantiomer of hydronaphthalenone led to the 8-hydroxyenone as the main product, i.e. again a product of allylic hydroxylation, which is quite disappointing since this product is easily accessible by (electro)chemical oxidation. However, the fungus *Mucor plumbeus* produced another hydroxylated metabolite, the 6α-hydroxyl derivative. Interestingly, the S-enantiomer of the starting substrate only led to the 8-hydroxyenone in this last case. Introduction of an additional methyl group on the carbon framework of the starting octaenone also led to different regioselectivities of the hydroxylation.

Hydrindane derivatives, which bear a five-membered B ring (instead of a six-membered ring in the decalones derivatives) have also been examined for biohydroxylation by the fungus *Rhizpous arrhizus*[92]. All the hydroxylations observed now occur at position 3, to the α,β-unsaturated ketone. This can be considered as

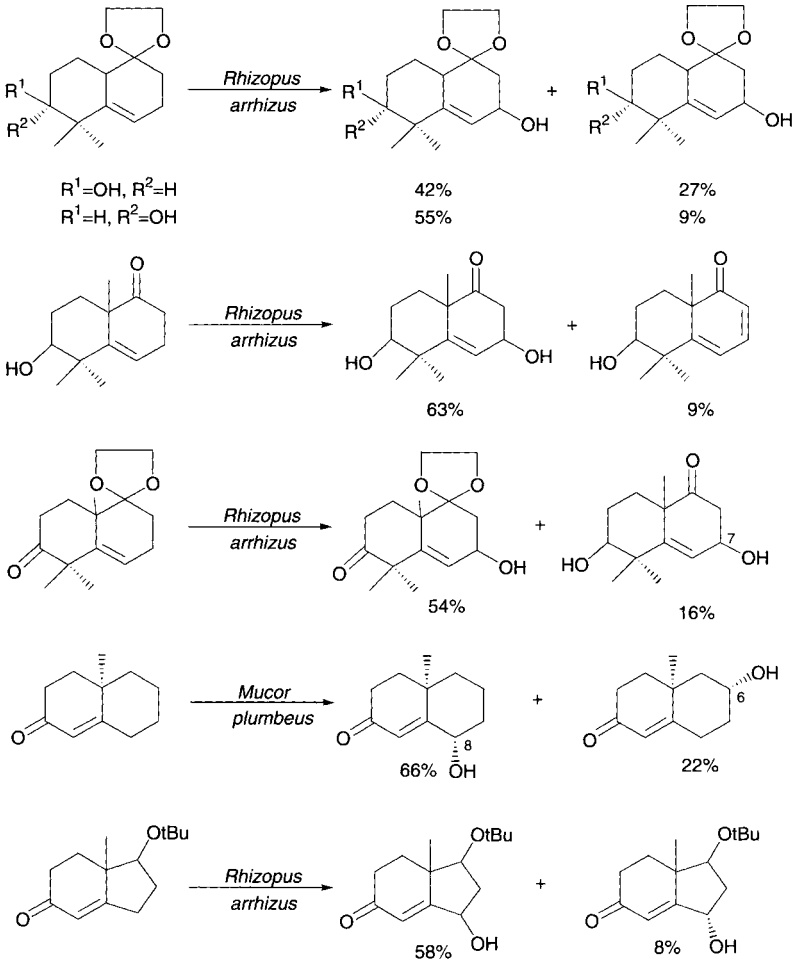


Figure 16.1-18. Microbiological hydroxylation of differently substituted octalones.

being formally analogous regioselectivity as compared to the results obtained on decalones. In this case, however, reactivity is identical in both antipodal series, and led almost quantitatively but with moderate or low stereoselectivity to the formation of the epimeric alcohols. Interestingly, these biohydroxylations prove to be complementary to lead tetraacetate oxidation of these substrates, which affords the 6-acetyl substituted products.

Structually much more complex molecules have also been submitted to regioselective enzymatic hydroxylation. Two such examples have been described involving milbemycin, a sixteen-membered macrolide which exhibits broad-spectrum insecticidal and acaricidal activity, and monensin, a carboxylic polyether antibiotic[93, 94]. Milbemycin (Fig. 16.1-19) was thus regioselectively hydroxylated at the 13β position (followed eventually by a C-29 hydroxylation) to afford the 13β,29-

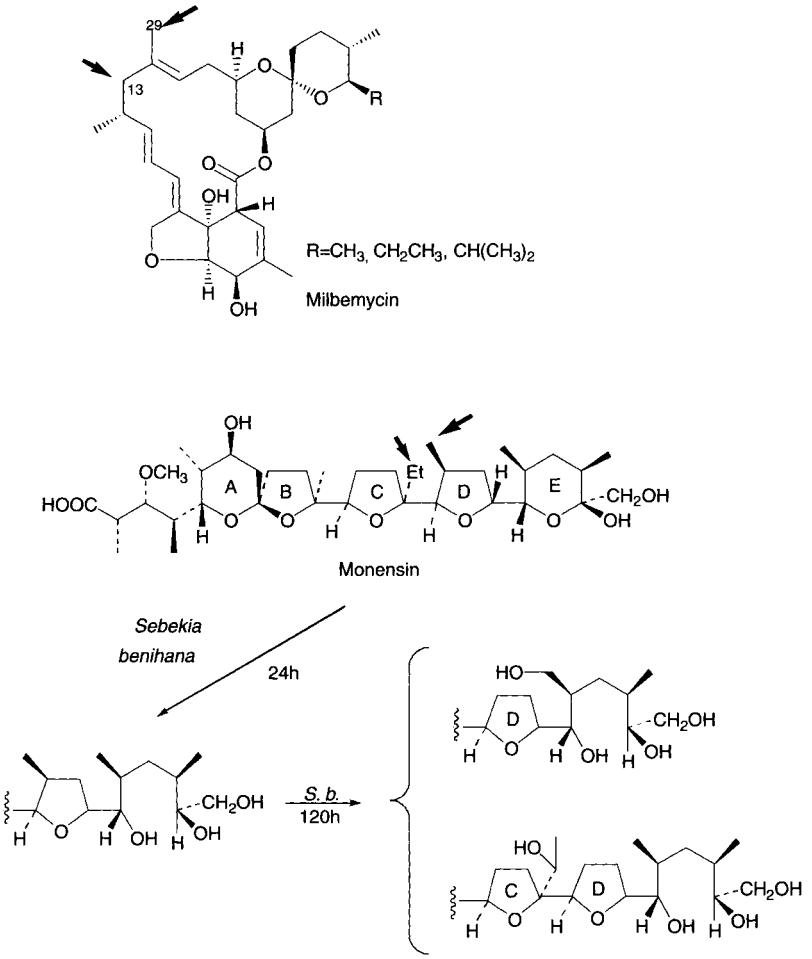


Figure 16.1-19. Regioselective hydroxylation of structurally complex substrates.

hydroxylated product by a strain isolated from solonized brown Mallee oil (collected in Adelaide in Australia) and identified as *Streptomyces cavourensis*. It is interesting to emphasize here the high regioselectivity observed for this hydroxylation of a rather complex and multifunctional compound.

Even more complex is the structure of monensin, a compound which has been extensively used as an anticoccidial agent for poultry and shown to improve the efficiency of feed utilization in ruminant animals. When submitted to a culture of *Sebekia bevihana*, monensin was first quantitatively converted by enzymatic reduction of the δ-hydroxy-ketone (which is in equilibrium with its hemiketal tautomeric form) and was regioselectively further hydroxylated at the C-29 methyl group as well as at the nearby ethyl group substituent[94].

All the previously described examples exemplify the ability of various monooxygenase enzymes to achieve, often with good to reasonable yields and interesting

regioselectivities, the hydroxylation of non-activated carbon atoms which are inaccessible using conventional chemistry. This thus allows one-step syntheses of these metabolites, which can in certain cases be of high enantiomeric purity. Another type of oxygenation reaction which is of interest is the stereoselective epoxidation of double bonds, the essential aim being in this matter the access to epoxides of high enantiomeric purity. This will be the subject of the following part of the discussion.

16.1.5
Epoxidation of Olefins

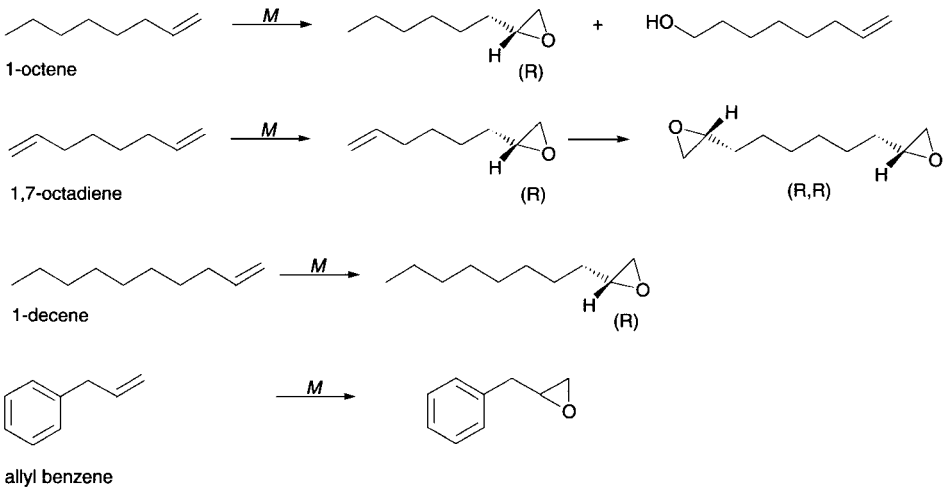
As discussed previously, monooxygenases provide highly activated oxygen intermediates that can oxidize a wide range of functional groups. One of the most studied among these has been the epoxidation of olefins[95–97]. This epoxidation is particularly interesting when applied to prochiral double bonds. Spectacular success has been obtained in the field of asymmetric chemical epoxidation, notably using Sharpless epoxidation catalysts for allyl alcohols and Jacobsen catalysts for aryl olefins, which has made epoxides key intermediates in the synthesis of chiral compounds. However, these chemical catalysts often have a limited "substrate" range, and biocatalysts can provide access to complementary structural motifs.

Without attempting to be exhaustive, we will try in this chapter to focus on results allowing us to directly oxidize olefins to their corresponding epoxide, using microbial cells. Other sources of monooxygenases, such as mammalian cells (microsomes) or plant cells, have been studied in this respect. However, these will not be considered in this review.

16.1.5.1
Epoxidation of Straight-Chain Terminal Olefins

One of the earliest observations implicating the formation of epoxides during microbial olefin metabolism was the report by Bruyn in 1954 that *Candida lipolytica* grown on 1-hexadecene produced 1-hexadecanediol (about 5% of the hydrocarbon consumed was accounted for as the diol)[98]. Molecular ^{18}O was shown to be incorporated into this diol, and the 1,2-epoxide was identified as one of the byproducts of this metabolism[99, 100]. Several further reports confirmed that enzymatic systems are able to achieve epoxidations. For instance, Van der Linden showed in 1963 that *Pseudomonas aeruginosa* grown on *n*-heptane and resuspended in a buffer solution produced the epoxide from 1-octene (Fig. 16.1-20)[101]. This led the authors to conclude that this epoxide was formed by enzymes already present in the alkane-grown cells and that epoxidation might be catalyzed by the same hydroxylases that would normally oxidize alkanes. A similar conclusion was reached by Maynert and coworkers[102], who demonstrated that epoxides are obligatory intermediates in the metabolism of simple olefins in rat liver microsomes.

However, the real breakthrough in the study of enzymatic epoxidations is due to Abbot and coworkers[103] and to May and coworkers[104], who established unequivo-



M: Pseudomonas oleovorans

Figure 16.1-20. Stereospecific epoxidation of straight-chain terminal olefins.

cally that epoxides are formed from terminal olefins by the bacterial strain *Pseudomonas oleovorans* (Fig. 16.1-20). They showed that 1-octene is epoxidized to 1,2-epoxyoctane of (*R*)-configuration (*ee* 70 %) or hydroxylated to 7-octen-1-ol. The 1,7-diene is exclusively epoxidized, affording 7,8-epoxy-1-octene, which can be further processed to the corresponding diepoxide[105]. It was shown later that this monoepoxidation was stereospecific, leading to the *R*(+)-7-epoxide showing an *ee* of about 80 %. Furthermore, the diepoxide was shown to be essentially of (*R*,*R*) configuration. This interestingly indicates that the configuration of the monoepoxide formed at one end of the molecule profoundly affects the stereochemical course of the reaction. Indeed, the authors showed that when starting from racemic monoepoxide, the diepoxide was essentially formed from the (*R*)-monoepoxide. Interestingly it was observed that olefins bearing an allylic (or homoallylic) hydroxyl were not epoxidized, but were converted instead to the corresponding saturated ketones.

One of the most useful characteristics of this work is the fact that these epoxides could be routinely produced at yields approaching (at best) 1 g L^{-1} after simple overnight shaking using whole-cell or even crude cell-free systems. Thus, these results clearly opened the way to a new type of biotransformation which should be very useful for organic synthesis.

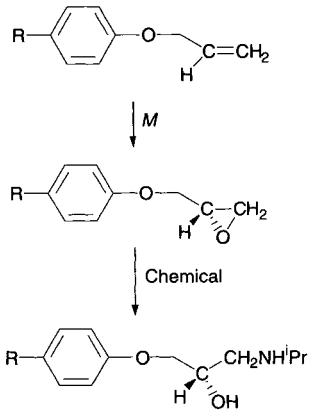
The enzymatic system involved in hydroxylation reactions of long-chain alkanes had been previously studied by Coon and coworkers, who isolated an enzyme system from *P. oleovorans* that catalyzes co-hydroxylation of alkenes and fatty acids[20, 106–115]. This was resolved into three protein components: rubredoxin (an iron-sulfur protein of molecular weight 19 000), an NADH-rubredoxin reductase (a flavoprotein of molecular weight 55 000) and an "ω-hydroxylase" (characterized as being a non-heme iron protein, with one iron atom and one cysteine per polypeptide

chain). Interestingly, it was shown that this same enzyme system is responsible for the conversion of terminal olefins to their corresponding 1,2-epoxides [104]. This leads to a competition between the two types of biotransformations, which results in a specific pattern for each type of substrate. Thus, further investigation demonstrated that this monooxygenase can produce epoxyalkanes with from six to twelve carbon atoms containing terminal alkenes. As a result of the influence of carbon chain length on epoxidation versus hydroxylation it was shown that hydroxylation predominates for the "*short*" substrates propylene and 1-butene, but that epoxidation activity falls off much less readily than hydroxylation for "*long*" substrates. For the "*medium*" length substrates, like for instance 1-octene, both reactions do occur. Thus, this substrate is epoxidized to 1,2-epoxyoctane or hydroxylated to 7-octen-1-ol, while for 1-decene epoxidation largely predominates. Interestingly, the epoxidation reaction exhibits a specificity far different from that expected for chemical reactivity. Indeed, terminal olefins are epoxidized exclusively even in the presence of more highly substituted (electron-rich) double bonds. Thus, cyclic and internal olefins were not epoxidized. This indicated that the substrate specificity pattern observed severely moderates the inherent reactivity of the activated oxygen species involved in these transformations. Methyl imidoesters as well as sodium cyanide were found to be inhibitors of enzymatic epoxidation, and the potency of a homologous series of imidoester inhibitors was examined.

In the reaction with dienes, 1,5-hexadiene to 1,11-dodecadiene were epoxidized while dienes with a smaller number of carbon atoms were hydroxylated to the corresponding unsaturated alcohols [116]. The reactivity was shown to be maximal for octadiene (leading to 0.3 to 0.4 g of diepoxyoctane per liter) and falls off rapidly as the carbon chain is shortened, but decreases only slightly as the chain is lengthened. In a further study, it was shown that a very efficient conversion of 1,7-octadiene to 7,8-epoxy-1-octene and 1,2–7,8-diepoxyoctane could be obtained by incorporating a high concentration of cyclohexane into the conventional fermentation medium. Thus, a 90% yield of product was achieved within 72 h, instead of a 18.5% yield in the absence of cyclohexane, when a 20% (v/v) amount of cyclohexane was used. Clearly, this is an early example of the use of organic solvents applied to microbial transformations [117].

A similar result was obtained later on using the 1-octene substrate itself as the organic phase (20% v/v), leading to comparable results (70% *ee*) [118]. Interestingly it also has been shown in the course of this work that, when *n*-hexadecane (which is not metabolized by the cells) is used as a solvent, racemic epoxide is enantioselectively degraded by the "ω-hydroxylation" enzymatic system of P. *oleovorans*, leading to an enrichment in (*S*)-1,2-epoxyoctane.

Further work by Wynberg and coworkers was aimed at even increasing the yield of 1,2-epoxyoctane using an optimized two-phase system and a cell renewal procedure [119]. Thus, yields up to 150 mg 1,2-epoxyoctane per mL 1-octene and up to 20–25 mg 1,2-epoxyoctane per mL culture was obtained. Some other substrates were tested in this optimized system. Of these, 1-decene was converted into (*R*)-1,2-epoxydecane (60% o.p.), while allylbenzene was converted to the corresponding epoxide. However, no effort was made to determine the absolute configuration and the optical purity of this product.



Organism *M*	Metropolol o.p. (%)
R. equi NCIB 12035	95.4
P. putida NCIB 9571	98
P. oleovorans AT CC 29347	98.4
P. aeruginosa NCIB 8704	98.8

R = $CH_2CH_2OCH_3$: Metropolol
R = CH_2CONH_2 : Atenolol (*M: P. oleovorans* o.p. = 97%)

Figure 16.1-21. Microbiological epoxidation as a way to optically pure β-blocker drugs.

All these results led to an interesting application for asymmetric organic synthesis. Thus, *P. oleovorans* has been used, among some other microorganisms, for stereospecific epoxidation of some arylallylethers into (+)-arylglycidyl ethers (Fig. 16.1-21). These intermediates were chemically converted into (*S*)-(–)-3-substituted-1-alkylamino-2-propanols, which are the physiologically active components of the β-adrenergic receptor blocking drugs. This method has been used to synthesize (*S*)-(–)-Metoprolol and (*S*)-(–)-Atenolol with enantiomeric purities of 95.4–98.8% and 97% respectively[120]. These applications are of great industrial interest, since it has been shown that (*S*)-(–)-Metoprolol is 270–380 times more active than its antipode[121].

Microorganisms screened for epoxidation activity were selected from bacteria belonging to the genera *Rhodococcus, Mycobacterium, Nocardia* and *Pseudomonas*. Species of *Pseudomonas* gave the best activities, but there were variations between the individual members, and *P. oleovorans* was the most active organism. The activity was further enhanced by carrying out the transformation in the presence of a cosubstrate such as glucose.

This pioneering work on microbial expoxidation of straight-chain terminal olefins has triggered several further studies aimed at preparing enantiopure epoxides via biotransformations. Thus, a number of alkene-utilizing microorganisms have been described in the literature. In the context of aliphatic substrates these efforts have been developed essentially along two lines: epoxidation of long-chain olefins and epoxidation of short (C_1-C_4) chain compounds. Thus, for instance, it was shown that *Corynebacterium equi* (IFO 3730) grown on *n*-octane is able to oxidize 1-hexadecene to give the corresponding optically pure (*R*)-(+)-epoxide (41% yield based on consumed substrate)[122, 123]. This strain also assimilated other terminal olefins and produced

the corresponding epoxides from substrates which have a carbon chain longer than fourteen, although in very low yields (less than 1%). Production of 7,8-epoxy-1-octene from 1,7-octadiene by non-growing *Pseudomonas putida* species using two-phase transformation has also been achieved[124]. Similarly, a gaseous hydrocarbon-assimilating microorganism *Nocardia corallina* B-276 grown on 1-alkenes (C_3, C_4 and C_{13}-C_{18}) was described as being able to produce the corresponding 1,2-epoxy-alkanes. One of the products, 1,2-epoxytetradecane, was shown to be optically active. Glucose-grown cells could also transform styrene and C_2-C_{18} 1-alkenes to their epoxyalkanes[125]. Similarly, production of epoxides from C_6-C_{10} 1-alkenes and styrene was shown to be enhanced by using *n*-hexadecane as an additional solvent, while this led to a decreased rate for epoxidation of longer chain 1-alkenes[126]. Epoxidation of unsaturated fatty acids such as palmitoleic acid by *Bacillus megaterium* has also been reported[127]. Here again, experiments indicated that epoxidation and hydroxylation were catalyzed by the same soluble cytochrome P450-dependent enzymatic system.

16.1.5.2
Short-Chain Alkenes

Short-chain alkenes are another type of substrates which have been studied for microbiological epoxidation during the last thirty years. In this context, an extensive study has been conducted by De Bont and coworkers in order to prepare epoxides from gaseous olefins. Thus, a *Mycobacterium* sp. (E 20) was isolated from soil and shown to excrete ethylene oxide when grown on ethylene[128, 129]. Studies carried out using $^{18}O_2$ showed that a monooxygenase was involved in these epoxidations, as proved by incorporation of only one ^{18}O into the product. Another *Mycobacterium* (Py 1) was also shown to achieve this reaction. Experiments were performed in a gas-solid reactor to prevent accumulation of the toxic ethylene oxide in the immediate vicinity of the biocatalyst[130]. An experimental set-up, allowing for automatic gas chromatography analysis of circulation gas in a batch-reactor system, was also described allowing on-line monitoring of the microbial oxidation of the gaseous alkenes propene and 1-butene (Fig. 16.1-22)[131]. Optimization was achieved by studying the influence of various organic solvents on the retention of immobilized cell activity[132]. High activity retention was favored by a low polarity in combination with a high molecular weight. Using chiral gas chromatography (at that time recently described by Schurig and Bürkle[133]), eleven strains of alkene-utilizing bacteria were screened with respect to the stereospecific epoxidation of propene, 1-butene and 3-chloro-1-propene. The results obtained showed that seven of these bacteria strongly resembled each other, in that they all produced 1,2-epoxypropane and 1,2-epoxybutane mainly in the (*R*)-form (93 and 85% *ee* respectively). Several of these strains were also able to epoxidize stereoselectively 1-chloro-2,3-epoxypropane, thus leading to the synthetically very useful (*S*)-epichlorohydrin (*ee* > 95%). Stereoselective epoxidation of 4-bromo-1-butene and of 3-buten-1-ol was similarly studied using three strains. The results showed that the epoxides were again obtained predominantly in the (*R*)-form but that their enantiomeric purity depended on both

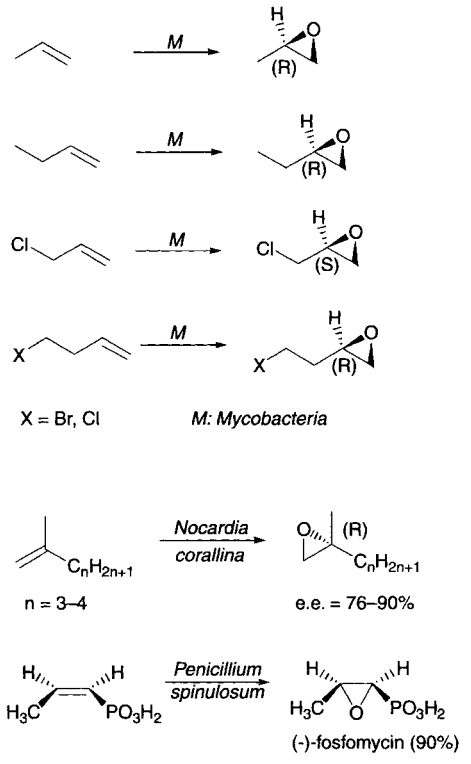


Figure 16.1-22. Short-chain alkene epoxidation.

the strain used and on the substrate studied[87]. Inactivation of the alkene oxidation enzymatic system by the produced epoxide was also investigated in view of setting up a biotechnological procedure for producing these epoxides[134]. Modeling the effects of mass transfer on the kinetics of propene epoxidation was also achieved by the same authors[135, 136], and they showed that product inhibition can be reduced by absorbing the epoxide in the gas phase in cold di-*n*-octyl phthalate[137].

In addition to the Mycobacterium species, several other strains have been reported to achieve epoxidation of olefins. Thus, three distinct types of methane-grown methylotrophic bacteria (*Methylosinus trichosporium, Methylobacterium capsulatus* and *Methylobacterium organophilum*) were shown by Hou and coworkers[138] to be able to oxidize terminal C_2 to C_4 *n*-alkenes to their corresponding 1,2-epoxides, which accumulated extracellularly. Results from inhibition studies indicated, as in the case of the previously discussed ω-hydroxylation system of *P. oleovorans*, that the same monooxygenase enzyme was responsible for the hydroxylation of methane and the epoxidation of alkenes. Further work achieved by the same group showed that whole cells of *Methylosinus* sp. CRL 31, immobilized by adsorption on glass beads, were able to convert propylene to propylene oxide for several hours until the reduced NAD cofactor was depleted. This could be regenerated by periodic addition of methanol. These authors also observed that attempts to immobilize the cells by covalent binding or entrapment in polyacrylamide gel led to complete loss of propylene

epoxidation activity[139]. However, no mention is made in this work of the enantiomeric purities of the obtained epoxides. Further studies carried out by Subramanian[140] revealed that these were nearly racemic compounds, and also that the major problem of these biotransformations was again product (epoxide) inhibition. More information about the reaction mechanism of the epoxidation achieved with whole-cell *M. trichosporium* was gained by Okura and coworkers[141], who showed that the configuration of the double bond was retained during the epoxidation of *cis*-2-butene. This result was further confirmed by studies of the epoxidation of 1,2-deuterated-*cis*-propene. A concerted insertion of oxygen was postulated to account for this result[142]. Oxidation of propylene to propylene oxide by *Methylococcus capsulatus* (Bath) was studied in order to optimize the biotransformation for a possible industrial production. However, the high rates obtained could only be sustained for 3–4 min before loss of biocatalytic activity occurred[143].

Similar results were obtained by Wyngard and coworkers[144] in the course of a study aimed at exploring how immobilization of the whole cells on solid supports would influence the rate and duration of the epoxidation of propylene by the strain *Nocardia corallina* B-276 initially isolated by Furuhashi and coworkers[125, 126]. Here again the results suggested that entrapment in a hydrophobic matrix might be a favorable system, but that loss of activity was quite rapid with time. The same *Nocardia* strain has been shown to be able to epoxidize branched chain terminal olefins in an asymmetric manner leading to (*R*)-epoxides showing optical purities of 76–90% depending on the chain length. These epoxides were used as chirons for further synthesis of prostaglandin ω-chains. The same strain was shown by these authors to be also able to epoxidize trifluoromethylethylene (75% *ee*)[145].

Some newly isolated *Xanthobacter* sp. were recently shown to be able to accumulate 1,2-epoxyethane from ethene or, when grown on propene, to accumulate 2,3-epoxybutane from *cis*- or *trans*-2-butene but with apparently low yields[146]. Similarly, *Rhodococcus rhodochrous*, a propane-oxidizing strain, was shown to produce 1,2-epoxyalkanes from short-chain terminal alkenes. Interestingly, its oxygenase enzyme appeared to be capable of tolerating high levels of product without inhibition[147].

Finally, a very useful and industrially interesting epoxidation which deserves special attention is the stereospecific epoxidation of *cis*-propenylphosphonate. Eighteen species of *Penicillium*, one of *Oidium* and one of *Paecilomyces* were found to effect this reaction, which affords directly (-)-fosfomycin, a broad-spectrum antibiotic. Using the strain *Penicillium spinulosum* MB 2843 at optimum culture conditions, a 90% efficiency (based on olefin charged, 0.5 g L^{-1}) was obtained after 6 days, leading to a product claimed to be optically pure[148].

16.1.5.3
Terpenes

Besides the extensive studies aimed at preparing optically active epoxides starting from short or long straight-chain alkenes, another area of investigation has been the microbiological epoxidation of various natural substrates, essentially in the terpene and steroid area. Interestingly enough, it appears that terminal olefins (and only

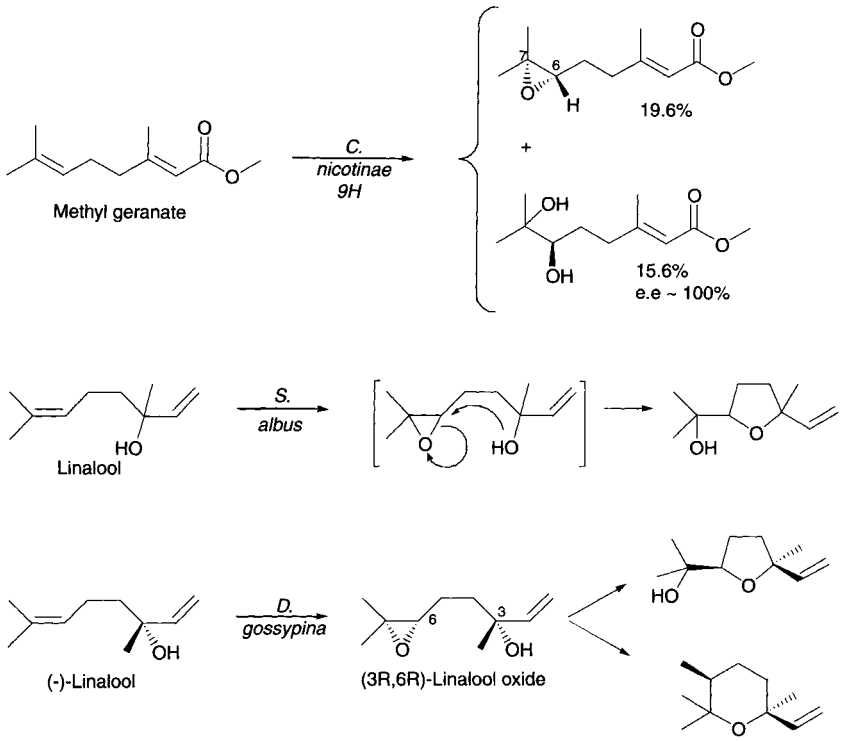


Figure 16.1-23. Some examples of olefinic terpene epoxidation.

these) are epoxidized almost exclusively by bacteria, and lead to accumulation of the corresponding epoxide in the culture. On the other hand, more substituted double bonds are often preferentially oxidized by higher organisms like fungi. The product is generally the corresponding vicinal diol arising from further metabolism (hydrolysis) of the primarily formed epoxide. Numerous publications describe microbial transformations of various terpenes[149, 150]. However, there are few cases of an accumulation of intermediates in sufficient amounts for further use in synthesis[151].

One of the first examples of such a transformation has been described by Marumo and coworkers (Fig. 16.1-23)[152]. Their investigations, aimed at preparing optically active insect juvenile hormone, showed that methylgeranate was metabolized by the fungus *Colletotrichum nicotianae*, leading to 19.6% of *S*(–)-methyl-6,7-epoxygeranate and to 15.6% of *R*(+)methyl-6,7-dihydroxygeranate after 9 h incubation. Longer incubation times (24 h) produced only the optically pure glycol with an isolated yield as high as 85%, showing that the first epoxidation step had to be stereospecific. Unfortunately, this analytical study was not pursued on a preparative scale, and no accurate results concerning the stereochemical and kinetical aspects of these interesting biotransformations have been described.

A similar microbial oxidation of the isoprene double bond has been studied by

Veschambre and coworkers starting from linalool[153]. Thus *Streptomyces albus*, a strain which synthesized nigericine, transforms each enantiomer of linalool, as well as the racemic compound, into a mixture (10–20% yield) of two diastereoisomeric linalool oxides. In this case, the epoxide formed primarily is trapped by an intramolecular cyclization. Based on the reported proportions of these products, one can deduce that the *ee* of the formed epoxide was about 35%. Further work achieved using several other microorganisms showed that *Beauveria sulfurescens* gave similar yields (15–20% analytical) of an equimolar mixture of linalool oxides[154]. *Botyris cinerea*, a fungus which participates in the formation of flavors in sweet wines, was also checked for linalool biotransformation. This led to several metabolites including linalool oxides, presumably arising from prior epoxidation of the olefinic bond[155]. Interestingly, these products were also detected in the *Carica papaya* fruit flavor, together with the diastereoisomeric epoxides[156]. It was also observed by Abraham and coworkers[157] that (–)-linalool is processed by *Diplodia gossypina* exclusively to a mixture of *trans*-(3*R*,6*R*)-linalool oxide and to the corresponding tetrahydropyran. These were proposed to arise by intramolecular cyclization of the intermediate 6(*S*)-epoxide. Some other similar substrates have been studied in the course of this study, but they generally led to low yield mixtures of products. Comparable results were obtained from linalool using the strain *Streptomyces cinnamonensis*[158]. Similar transformations were observed starting from 2-methyl-2-heptene-6-one[159]. However, because of the number of metabolites formed and the low yields obtained, these biotransformations cannot be usefully employed for organic synthesis.

Myrcene and *trans*-nerolidol were also shown by Abraham and Stumpf to be transformed by two fungi (*Diplodia gossypina* and *Corynespora cassiicola* respectively) into a mixture of several products including vicinal diols arising from oxidation of the isoprenyl double bond. These were shown to be further degraded, presumably via an acyloin-splitting mechanism[160]. During the course of the fermentation, the diol occurred at first in the culture medium followed by the nordiols and the trialcohols. So, the formation of these compounds from diols seemed to be very likely. Some other related substrates were also studied in the same context, and it was shown that both strains revealed a pronounced and almost opposite substrate selectivity. Much more impressive is the result obtained by the same group[161],who conducted a broad screen of 800 various microorganisms using both the (*S*)(–)- and the (*R*)(+)-limonene enantiomers as a starting substrate, as well as some other terpenes which were tested with the best suited strains (Fig. 16.1-24). The most interesting results were observed with *Diplodia gossypina* (ATCC 10936), which afforded 380 mg of a diol which was found to be the (1*R*, 2*R*, 4*S*)-8-*p*-menthen-1,2-diol from 1 g of (*S*)(–)-limonene. Similarly, *Corynespora cassiicola* (DSM 62474) was described to yield 1.1g of (1*R*, 2*R*)-3-*p*-menthen-1,2-diol from 1.8g of α-terpene. (*R*)(+)-limonene was shown to afford (1*S*, 2*S*, 4*R*)-*p*-8-menthene-1,2-diol.

Because of the interest of these products in flavor chemistry, the preparative-scale transformation of this enantiomer by the fungus *Diplodia gossypina* has been undertaken: thus 1300 g were transformed, yielding 900 g of the (1*S*, 2*S*) diol showing high optical purity[162]. Interestingly, these strains convert the substrates fast with only negligible amounts of side products. Also, it is noteworthy that the

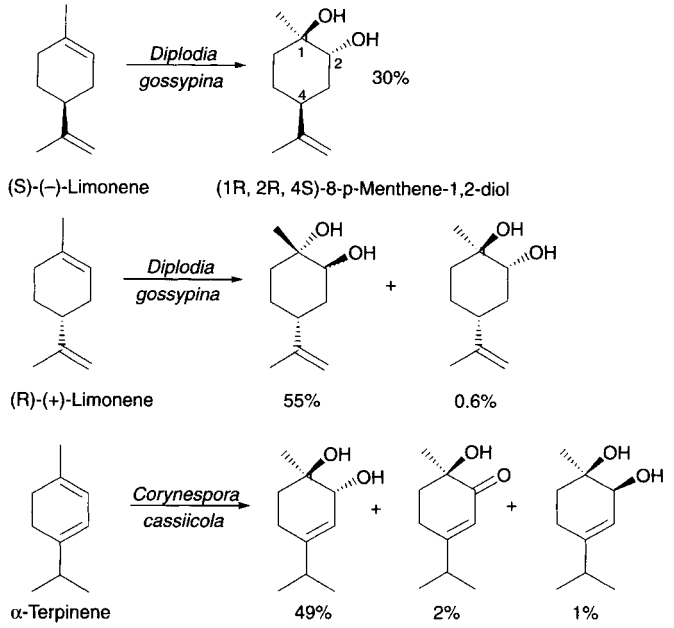


Figure 16.1-24. Stereoselective oxidation of monocyclic terpenes.

obtained diols are almost exclusively of *trans* configuration. No indication is provided concerning the determination and the values of the obtained products' optical purities. It was suggested that these *trans*-diols were formed via an intermediate epoxide, which could be further cleaved enzymatically to the obtained diols. Surprisingly, both these microorganisms were shown not to attack 3,3,5,5-tetramethyllimonene[163]. However, geranylacetone, nerylacetone, *trans*-nerolidol, *cis*-nerolidol, farnesol and 2,5-dimethyl-1,3-hexadiene were transformed by these strains to the corresponding glycols in yields of up to 70%[164, 165] and interesting optical purities of up to 98%. Using (+)-*trans*-nerolidol as a substrate, the strain *Nocardia alba* DSM 43 130 was shown to be lacking an epoxide hydrolase, thus leading to a 27% yield of the corresponding (*S*)-epoxide which accumulates in the culture medium[157].

Also, the ability of the monensin-producing organism *Streptomyces cinnamonensis* to convert the *cis* and *trans* isomers of nerolidol has been investigated[158]. However, here again this led to a low-yield mixture of several products.

Much more useful in that sense are the results obtained by Furstoss and coworkers in the course of their study of biooxygenation of geraniol derivatives (Fig. 16.1-25). Indeed, it has been described in a first paper that, if the *N*-phenylcarbamate of geraniol is used instead of geraniol itself, its transformation by the fungus *Aspergillus niger* leads to a 49% isolated yield of the 6,7-dihydroxylated product. Moreover, this diol proved to be of (6*S*) absolute configuration and was shown to possess an enantiomeric excess of about 95%[76, 166]. This diol, which is a very versatile substrate for further organic synthesis, can thus be obtained without problem in

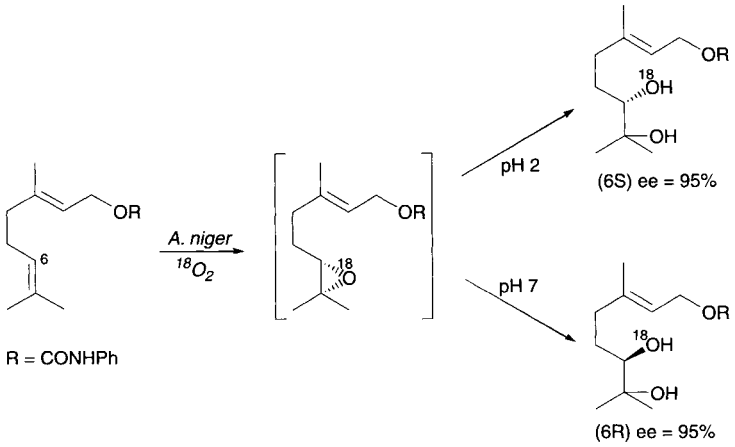


Figure 16.1-25. Stereoselective pH-dependent oxidation of geraniol *N*-phenyl carbamate.

gram-scale quantities (1 g substrate treated for 36 h in 1 L culture afforded 550 mg pure diol). Further work aimed at exploring the influence of the culture conditions showed that a unique stereochemical control could be achieved simply by modulating the pH of the medium. Thus, although when the culture was at pH 2 the diol of (*S*)-configuration was obtained, at pH 6–7 the diol of opposite (*R*) absolute configuration was isolated in similar yields and with an *ee* again as high as 95%. This interestingly showed that the fungus *A. niger* not only is able to convert the substrate across the pH 2–7 range, but that the (6*S*)-epoxide must be the primarily formed metabolite. This can then be further hydrolyzed in acidic medium (following the classical acid-catalysis mechanism) to afford the (6*S*)-diol or, at pH 6, be hydrolyzed enzymatically to the (6*R*)-diol by attack on the less substituted oxirane carbon atom[167]. Experiments conducted in the presence of ^{18}O confirmed this hypothesis. When the incubation was carried out at pH 2, the distribution of the ^{18}O label in the obtained diol was 95% on C-6 and 5% on C-7. This ratio was inverted at pH 7. These results show clearly that, whatever the pH, molecular oxygen is involved in these oxygenations but only one labeled oxygen atom is incorporated into the diol, leading to an epoxide which is differently hydrolyzed, depending on the pH of the medium.

Very interestingly as far as organic synthesis is concerned, these biooxygenations can be conveniently performed on a scale of several grams (5 g), thus allowing easy preparation of either enantiopure diol. These can be conveniently used as "*chirons*" for the synthesis of various natural or non-natural products. For instance they can be cyclized to the optically pure linalool oxides[168] or the corresponding tetrahydropyranols[169].

Biooxygenation of some other similar compounds, i.e. 7-geranyloxycoumarin, citronellyl *N*-phenylcarbamate and sulcatol *N*-phenylcarbamate were studied (Fig. 16.1-26)[170–172]. The reaction was shown to be operative in all these cases, leading, for instance to either enantiomer of marmin (a member of the umbelliferone family). Moreover, this result opens the way to an easy preparation of either

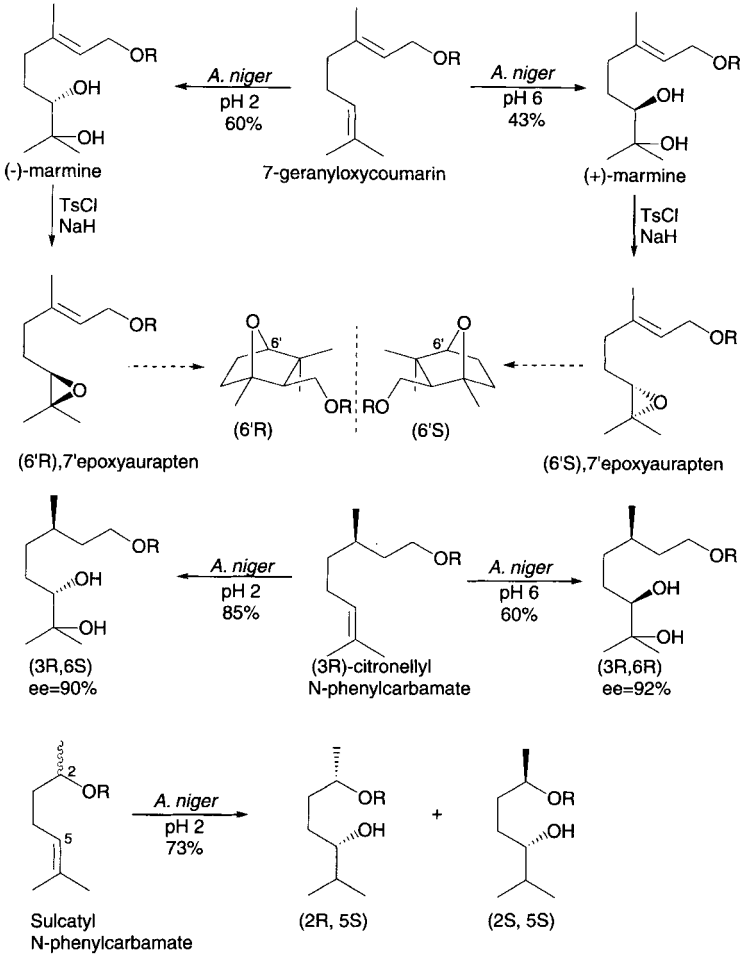


Figure 16.1-26. Application of the pH-dependent oxidation of geranyl derivatives to the synthesis of some natural products.

enantiomer of 6',7'-epoxyaurapten and of 3',6'-epoxyaurapten, both these compounds being natural products isolated from various sources. Similar results were obtained from both commercially available citronellol enantiomers, leading to the corresponding diols showing *ee*'s as high as 90 and 92%.

Bioconversions conducted at pH 2 on racemic sulcatol *N*-phenylcarbamate led to a 73% yield of a 1/1 mixture of the two expected diastereoisomeric diols, which can be readily separated by flash chromatography. They both show *ee*'s > 95%, indicating that the first (epoxidation) step again occurred in a highly stereospecific manner. Interestingly in this case, it was also possible to avoid hydrolysis of the intermediate epoxide by changing the preculture conditions and performing the reaction at neutral pH. This intermediate can thus be obtained directly with high enantiomeric purity. Using this chiron allows the four-step synthesis of optically pure pityol, a

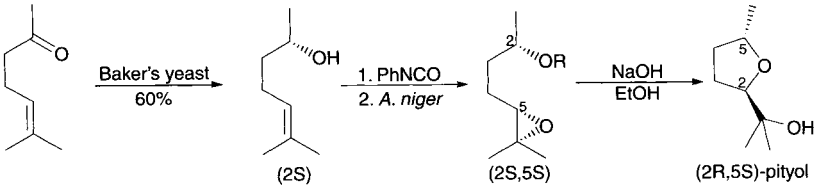


Figure 16.1-27. A four-step synthesis of (2*R*,5*S*)-pityol using microbiologically mediated steps.

male-specific attractant of the bark beetle *Pityophtorus pityographus*. Thus, prochiral 6-methyl-hept-5-en-2-one was reduced with baker's yeast to the corresponding alcohol (60% yield, 98.5% *ee*). This was converted to its *N*-phenylcarbamate, which was subsequently subjected to epoxidation using *A. niger*, thus affording a 50% preparative yield of the corresponding enantiopure epoxide. In a final step, treatment of the epoxycarbamate with an alcoholic NaOH solution led to the natural (2*R*,5*S*)-pityol (7.5% overall yield, 100% *ee*, 98% *de*) (Fig. 16.1-27).

16.1.5.4
Cyclic Sesquiterpenes

Various cyclic sesquiterpenes have also been studied in order to explore the possibility of achieving their microbiological transformations. Very often these were shown to lead to epoxidation processes when one (or several) double bonds were present in the starting substrate (Fig. 16.1-28).

Thus germacrone, which is thought to be the precursor of a variety of bicarbocyclic sesquiterpenoids, was shown to be transformed by the fungus *Cunninghamella blakesleena*. This led primarily to regio- and stereoselective epoxidation of one of the intracyclic double bonds of this prochiral triene, thus affording two epoxides. The third product isolated from this experiment was due to subsequent epoxidation of the remaining intracyclic double bond. Interestingly, the exocyclic olefinic bond conjugated to the carbonyl function appeared resistant to oxidation [173].

Valencene, another olefinic sesquiterpene, has been studied in the same context using microorganisms isolated from soil [174]. It was observed that these biotransformations led in reasonable yields to a mixture of three main metabolites, including an epoxide and nootkatone, an interesting flavoring compound.

The microbial transformation of humulene, a substrate showing a structure similar to that of germacrone, was studied by Abraham and Stumpf using a screen of about 300 strains [175]. This led the authors to select the fungi *Diplodia gossypina* and *Chaetonium cochlioides* for preparative scale experiments. It was thus observed that the main reaction path starts with the epoxidation of the 1,2-double bond, as shown by direct biotransformation of this monoepoxide obtained by chemical synthesis. This is then further oxidized to yield a multitude of products including diepoxides and hydroxy-epoxides (Fig. 16.1-28).

Comparable results were obtained from caryophyllene, a compound similar to humulene. Again, the biotransformation of this substrate with cultures of *Chaeton-*

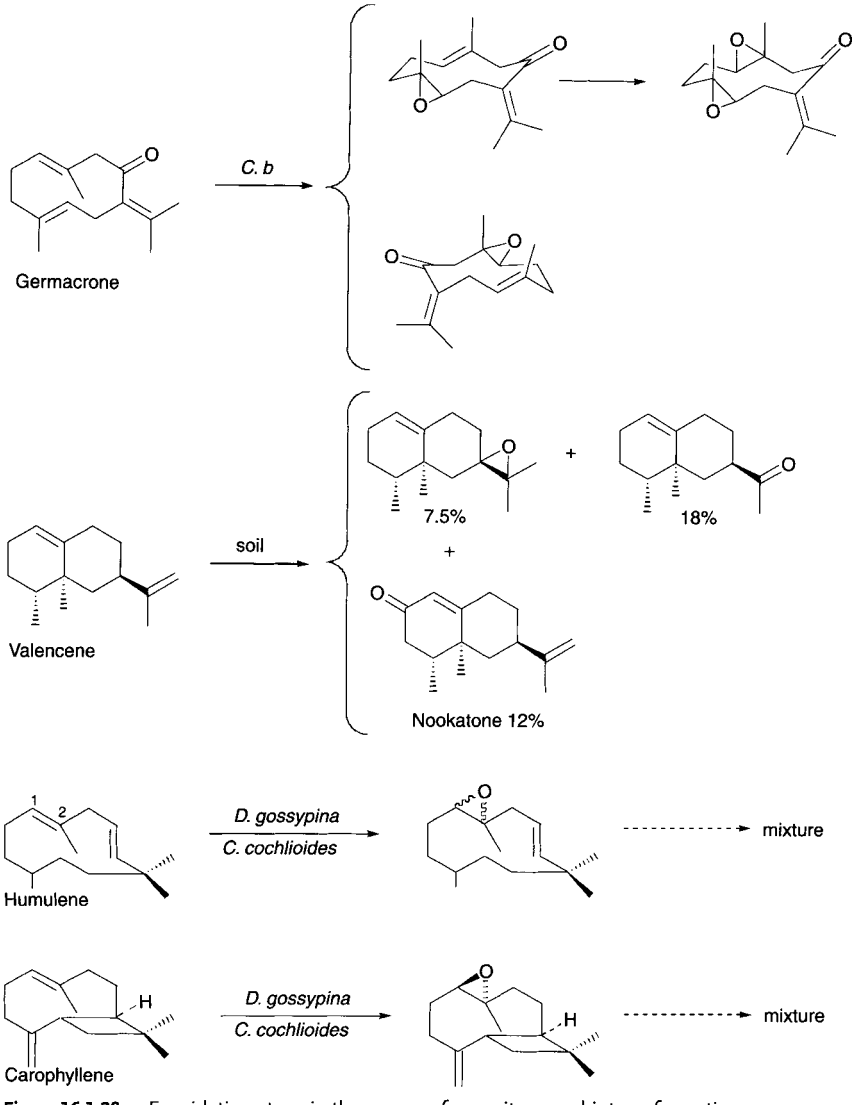


Figure 16.1-28. Epoxidation steps in the course of sesquiterpene biotransformations.

ium cochlioides as well as of *Diplodia gossypina* give a broad spectrum of products, resulting from an initial epoxidation of the 1–2 double bond followed by additional epoxidation or hydroxylation processes (Fig. 16.1-28) [176, 177].

16.1.6
Conclusions, Current and Future Trends

This review has illustrated the very broad range of biohydroxylations and epoxidations that can be achieved using monoxygenase enzymes. In fact, one can propose

that almost all organic compounds are potential substrates for these enzymes. Since each substrate can lead to many different oxidized products, the range of compounds that can be generated is clearly enormous.

Finding new enzymes with novel substrate specificities and selectivities of reaction has in the past been achieved by screening organisms and substrates and has very much been down to good luck. Current and future work is focused on finding methods to make this process faster and more rational and predictable. This is now possible because of new technologies in genetics, molecular biology and structural biology, of which a few highlights are discussed below.

More and more P450 monoxygenases have been sequenced and cloned into heterologous expression systems. This can have the advantage of higher turnover yields because of higher expression of the enzyme in the host or because higher cell mass can be obtained when using easy growing organisms such as *E. coli* as hosts[178, 179]. Heterologous expression can also overcome problems of loss of product because of further metabolic degradation[180] as in the case of the *alk* gene of *P oleovorans*. Such expression systems also allow the facile generation of chimeric enzymes and mutants with more desirable biocatalytic properties, such as increased activity towards a particular substrate[181, 182]. Some of the popular organisms for biohydroxylations such as *Beauvaria bassiana* also might contain several endogenous P450 enzymes that can interfer with selectivity of one enzyme and make predictions of reactions very difficult[183].

The rapid emergence of whole genome sequences has made a major impact on the study of P450 monooxygenases[184], since they are often easily identifiable by small conserved consensus sequences, in particular around the heme binding site. We now know that *Mycobacterium tuberculosis* contains probably twenty different P450 monoxygenases; *Bacillus subtilis* contains seven. The *a priori* prediction of substrate specificity and selectivity from gene sequence is at the moment impossible and presents a great challenge to the researcher. However, there has been some success in prediction of substrate specificity by "in silico screening" based on available three-dimensional structures of P450-monooxygenases[185]. Thus, substrate docking algorithms were used to predict substrate suitability for P450cam and its L244A mutant from a library of commercially available compounds.

The most practical way of using P450-based biocatalysts is still in whole-cell systems, because of cofactor requirements and problems with enzyme stability. However, some P450 monooxygenases, such as the P450cam, can be isolated in sufficient quantities and reconstituted for cell-free preparative scale biotransformations[182]. This might be particularly useful for substrates that cannot penetrate cell walls, are toxic to the organism or are unstable in the organism. One solution for overcoming co-factor requirements might be the use of electrochemical methods, and is has indeed been shown that P450cam can be immobilized on an electrode and can take up electrons from the electrode[186].

Another novel area of intense research is the application of mutagenesis (random and directed) to obtain desired changes in substrate specificity. Thus P450cam, which is highly selective for camphor and closely related analogs, was subjected to site-specific mutagenesis, changing the tyrosine in position 96 to a phenylala-

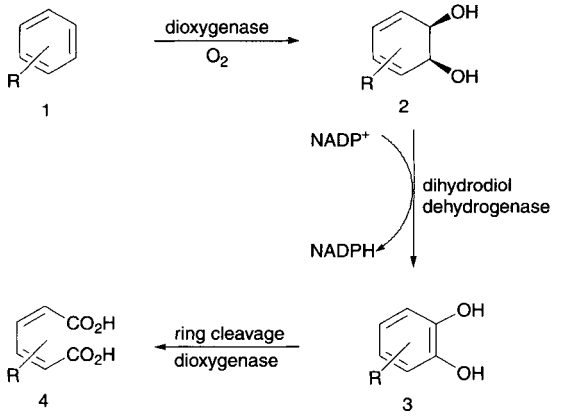
nine[182], which resulted in about a 20-fold increase in the reactivity towards naphthalene. The P450 monooxygenase was independently subjected to random mutagenesis by Arnold and co-workers[187], and mutants were screened for increased activity towards naphthalene. Similar improvements to those observed by specific mutagensis were obtained. However, interestingly, the mutations that were found to be responsible for improved activity were not at position 96, but were distant from the active site of the enzyme. Such a "directed evolution" approach has great promise in quickly generating desired biohydroxylation catalysts, provided that a suitable screening system for the product can be found. The method has also been recently used by the same group on P450BM3[188].

In conclusion, the application of biocatalysts in biohydroxylations and epoxidations is rapidly expanding in terms of practicality, substrate range and selectivity. A vast diversity of P450 genes is generated by genomics programmes and mutagenesis. Methods for screening such oxidation catalysts are becoming more rapid, and one can forsee a future where designer biooxidation catalysts, tailored for a specific substrate and even for selectivity of reaction, can be generated within short time spans using a combination of rational and screening methods.

16.1.7
Cis Hydroxylation of Aromatic Double Bonds

16.1.7.1
Introduction

The microbial dioxygenation of aromatic compounds **1** has been known for over thirty years through the pioneering efforts of D. Gibson et al., who characterized the metabolic pathway of toluene degradation[189]. In lower organisms, the chiral *cis* glycol intermediates **2** are rapidly oxidized by dihydrodiol dehydrogenase, involving rearomatisation to the diol **3**, which is further oxidized by ring cleavage dioxygenase to give dicarboxylic acid **4**, which can be channeled into the organism's normal metabolic pathways (Scheme 16.1-1) [190–192].



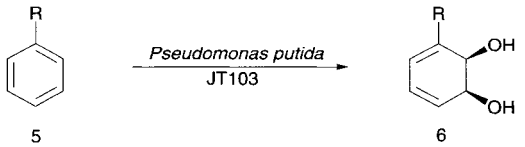
Scheme 16.1-1. Oxidative degradation of aromatic compounds by microorganisms.

The use of certain strains of *Pseudomonas putida*, most notably the mutant 39 D with blocked dehydrogenase activity[193], allows accumulation of the chiral glycols in the fermentation medium associated with high stereospecificity while the substrate tolerance remains high with respect to ring substituents. The enzymology of dioxygenases has been surveyed[190], and refinement of the mechanistic details of the dioxygenases continues[194], but only those enzymes and applications of relevance to the preparative biotransformations will be considered here.

16.1.7.2
Preparation of *cis* Dihydrodiols

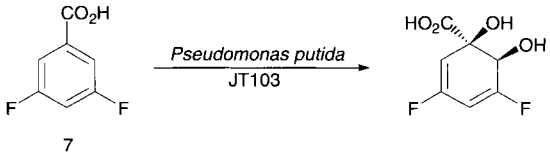
An impressive number of substituted aromatic compounds **5** have been converted by mutant strains of *Pseudomonas putida* into the corresponding chiral *cis* glycols **6** with often excellent stereoselectivity[195, 196]. The remarkable substrate range and selectivity of this dioxygenase system for the aromatic ring have been demonstrated by the conversion of a series of substituted benzenes and of alkenyl benzenes with the side chain double bond being left intact (Scheme 16.1.2) [197, 198]. An analogous product was obtained from *para*-fluorotoluene, but the dihydrodiols from *para*-chloro- and *para*-bromotoluenes were found to be racemic[199]. Unlike the substrates shown in Scheme 16.1-2, benzoic acid, toluic acid, and their halogenated analogs, for example **7**, undergo enzymatic dioxygenation by *Alcaligenes eutrophus* B 9 and two strains of *Pseudomonas*, for example JT 103, mainly at the 1,2-position (Scheme 16.1-3)[200]. However, with other strains of *Pseudomonas putida*, for example JT 106, enantiospecific *cis* 2,3-dihydroxylation is possible, too[201].

The structure of the substrates is not necessarily restricted to monocyclic aromatic compounds such as those shown in Scheme 16.1-2. The dioxygenase activity of *Pseudomonas putida* and *Beijerinckia* species has been used exclusively for the synthesis of *cis* dihydrodiols from polycyclic[202] and heterocyclic[203] derivatives. Such products have been obtained from naphthalene, anthracene, phenanthrene, benz[*a*]pyrene, benz[*a*]anthracene, and methylsubstituted benz[*a*]anthracenes, and

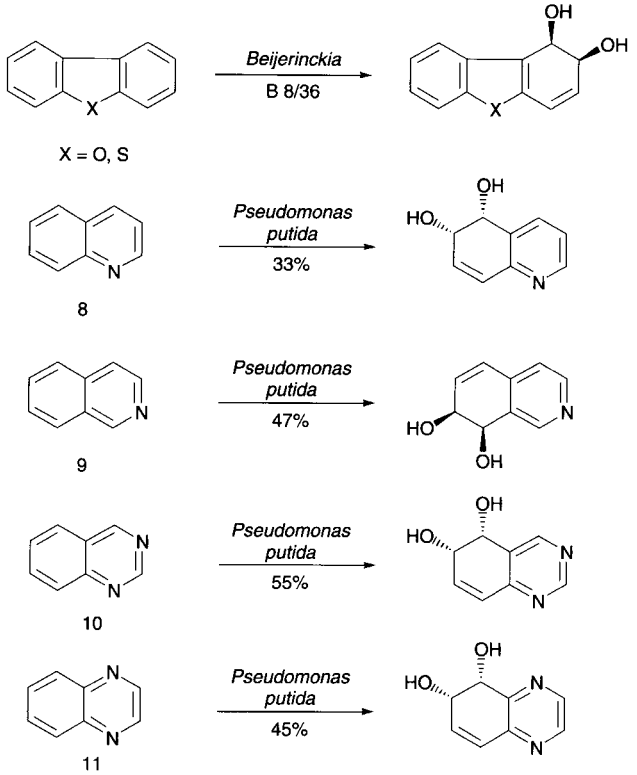


R = H, Me, Et, *n*Pr, *n*Bu, *t*Bu, EtO, *n*PrO, halogen, CF_3, Ph, $PhCH_2$ PhCO, $CH(OH)CH_3$, $COCH_3$, CH_2=CH, CH_2=$CHCH_2$, CH_3CH=CH CH3C=CHCH3, HC≡C, CF_3, CN, CO_2R, $SiMe_3$

Scheme 16.1-2. Synthesis of *cis* diols by *Pseudomonas putida*.



Scheme 16.1-3. *Cis*-hydroxylation of aromatic carboxylic acids by *Pseudomonas putida* JT 103.

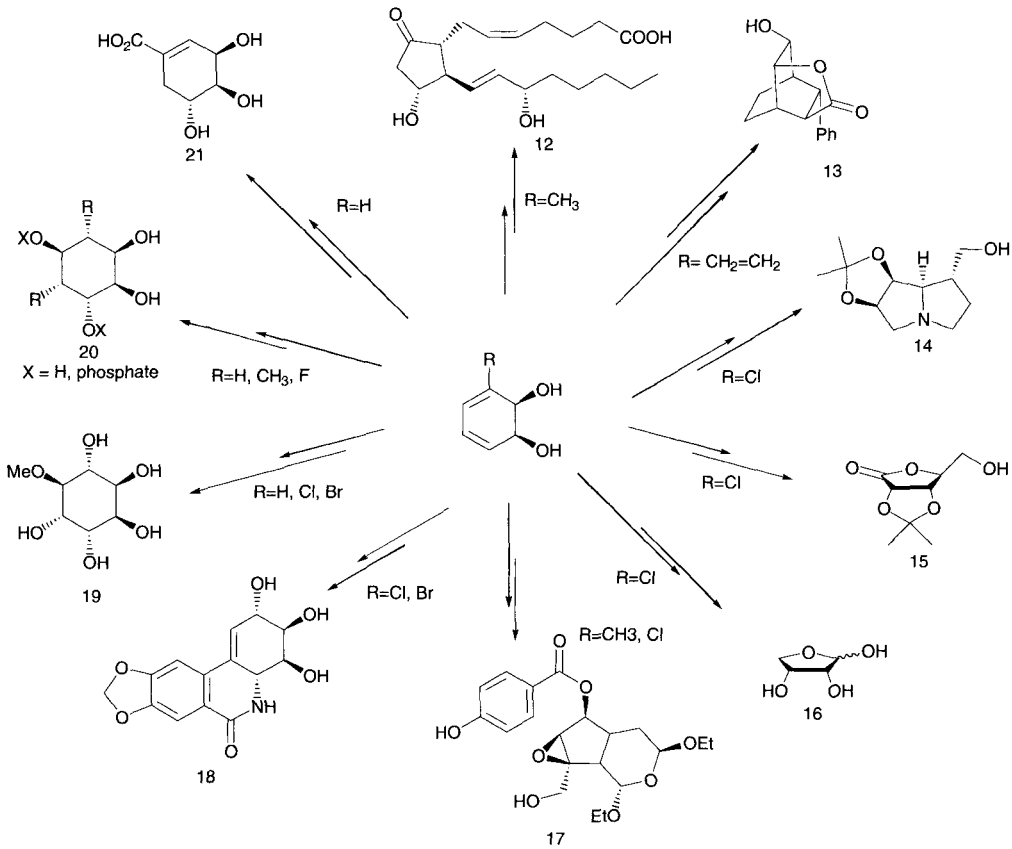


Scheme 16.1-4. Dioxygenation of condensed aromatics by *Beijerinckia* and *Pseudomonas putida*.

many of the enzymes responsible have been identified and characterized[191]. Benz[*a*]anthracene, for example, is converted to three *cis* dihydrodiol regioisomers by *Beijerinckia* B 8/36[204]. This organism has also been reported to produce dihydrodiols from dibenzofuran[205] and dibenzothiophene (Scheme 16.1-4)[206]. The ability of a *Pseudomonas putida* mutant to metabolize heteroaromatic compounds is demonstrated by the bioconversion of quinoline **8**, isoquinoline **9**, quinazoline **10**, and quinoxaline **11**[207]. Attack occurred exclusively in the carbocyclic ring (Scheme 16.1-4).

The impact of the genetic revolution has been greater in the area of dioxygenase-catalyzed reactions than in many other areas of bioconversion, largely because of the bacterial origin of the enzymes concerned. The bacterial oxidation of aromatic double bonds to *cis* diols in an enantiospecific manner leads to highly interesting synthons for organic chemistry. For example, the diene may be subjected to Diels-Alder reactions, as well as Michael-type addition reactions. Alternatively, oxidative cleavage of the cyclohexadiene ring leads to open chain products, which further react to yield cyclopentanoids. The large synthetic potential of chiral *cis* glycols is illustrated in Scheme 16.1-5.

T. Hudlicky et al. efficiently synthesized the prostaglandin PGE_2 **12** through an oxidative ring cleavage of the methyl-substituted diol **6** (R = CH_3)[208], the vinyl



Scheme 16.1-5. Syntheses of natural products from substituted cyclohexadienediols.

derivative **6** (R = CH = CH_2) was used for the construction of the plant metabolite (-)-zeylena **13**[209], and the chloro-substituted diol for the synthesis of the alkaloid trihydroxyheliotridane **14**[210] and the carbohydrates L-ribonolactone **15**[211] and D-erythrose **16**[212]. Hudlicky et al. also prepared the sesquiterpene specionin **17**[213], an antifeedant to the spruce budworm, and the narcissus alkaloid lycoricidine **18**[214] in only nine steps.

Biologically active polyols like pinitol **19**[215, 216], D-myo-inositol **20**[217], conduritol C[218] and conduritol E[219] were obtained from diol **6** in both enantiomeric forms in only a few steps using this approach.

Futhermore C. R. Johnson et al. synthesized (–)-shikimic acid **21**, the biosynthetic precursor of the benzene moiety of aromatic amino acids[220].

In the case of the cyclohexadienediols **6**, the current development promises to complement the traditional and rather arduous use of carbohydrates as starting materials from the chiral pool. The popularity of diol-based methods will, therefore, be directly proportional to their ready commercial availability and to the operational

ease of their transformations for the stereocontrolled introduction of further functionalities.

Several supply houses are now providing some simple chiral diols of type **6**, and further applications will assuredly follow.

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16.2
Oxidation of Alcohols

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16.2.1
Introduction

The enzymatic oxidation of alcohols is catalyzed by different oxidoreductases. Here, examples of dehydrogenases, oxidases, and peroxidases are discussed. Single enzymes were selected based on representative or demanding reactions that are catalyzed, or because of interesting reaction engineering solutions applied. Reactions catalyzed by whole microbial cells are described in a separate chapter. A focus is put on presenting or introducing enzyme catalysts and their substrate spectra in order to give the reader a basis for designing his or her own, new reactions with sterically or electronically similar compounds or with such compounds which are compatible with a certain reaction mechanism.

Biocatalysis usually exploits advantageous features of enzymes such as chemoselectivity, regioselectivity, enantioselecivity and substrate spectrum of a certain broadness as depicted in Fig. 16.2-1. These points are addressed in examples in the following chapters.

16.2.2
Dehydrogenases as Catalysts

16.2.2.1
Regeneration of Oxidized Nicotinamide Coenzymes

Regeneration of $NAD(P)^+$ from NAD(P)H is a redox reaction involving the transfer of two electrons and a proton (successively or at once as hydride ion H^-) to a suitable acceptor. Most commonly these acceptors are carbonyl functions, molecular oxygen or the anode. Apart from a few exceptions the direct hydride transfer is slow or disadvantageous so that catalytic procedures have to be applied. Here we selected representative examples to give an overview. Excellent review articles are available, too[1–3, 10].

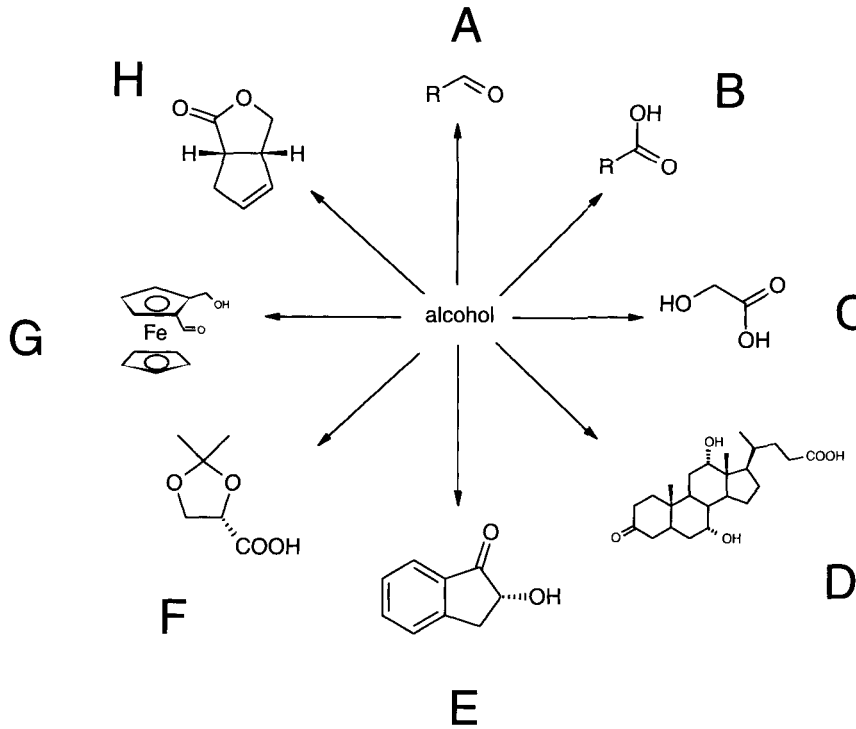


Figure 16.2-1. Enzyme-catalyzed oxidations of alcohols. Reactions are grouped according to the feature mainly exploited in the preparative application. A-C: Chemoselectivity (e.g. Sects. 16.2.2.3, 16.2.2.6, and 16.2.2.11); C, D: Regioselectivity (e.g. Sects. 16.2.2.9, 16.2.2.10, and 16.2.3.4); E, F: Enantioselectivity (e.g. Sects. 16.2.5.2 and 16.2.6.4); G: Non-natural substrates (e.g. Sect. 16.2.2.3); H: Complex structures from simple starting materials (e.g. Sect. 16.2.2.3).

16.2.2.2
Dehydrogenases as Regeneration Enzymes

Today, the utilization of a dehydrogenase-catalyzed reduction reaction is still the most widespread approach for the regeneration of oxidized $NAD(P)^+$. Its principle is displayed in Fig. 16.2-2.

Most commonly, alcohol dehydrogenase (E.C. 1.1.1.1) from yeast (YADH), horse liver (HLADH), or *Thermoanaerobium brockii* (TBADH) as well as glutamate dehydrogenase (E.C. 1.4.1.2.) or lactate dehydrogenase (E.C. 1.1.1.27) are used for $NAD(P)^+$ regeneration (Table 16.2-1). Thus, the reduction equivalents are transferred to an aldehyde or ketone as terminal electron acceptor yielding the corresponding alcohols.

The drawbacks of this approach result from the necessity to use a second enzyme, whose optimal reaction conditions may differ significantly from those of the actual production enzyme, and the presence of cosubstrates and coproducts. Furthermore,

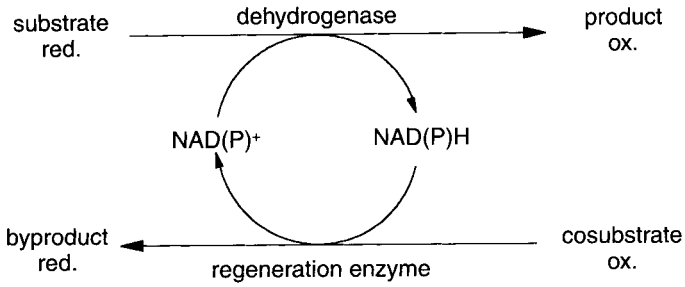


Figure 16.2-2. Enzymatic regeneration of oxidized NAD(P)$^+$.

Table 16.2-1. Comparison of commonly used dehydrogenases for NAD(P)$^+$ regeneration.

Regeneration enzyme	Cosubstrate/ coproduct	Specific Activity [U mg^{-1}]	Stability	Coenzyme	E'$_0$ [V] vs. NHE[a] [1]
YADH	Acetaldehyde/ ethanol	300	Low, sensitive to O_2	NAD$^+$	– 0.199
TBADH	Acetone/ isopropanol	30–90	Thermostable	NADP$^+$	– 0.286
Glutamate DH	α-Ketoglutarate/ glutamate	40	High	NAD$^+$ and NADP$^+$	– 0.121
Lactate DH	Pyruvate/lactate	1000	High	NAD$^+$	– 0.185

a NHE: normal hydrogen electrode.

1 H. K. Chenault, G. M. Whitesides, *Appl. Biochem. Biotech.* **1987**, *14*, 147–197.

the thermodynamical driving force is low because the formal redox potential of the cosubstrate/coproduct couple is often close to that of the NADH/NAD$^+$ couple. Some of these problems can be addressed using the following regeneration concepts:

16.2.2.2.1 Enzyme-Coupled Regeneration

Since dehydrogenase catalysis is reversible, the production enzyme can be used to perform the regeneration reaction of NAD(P)$^+$ using a suitable cosubstrate as electron acceptor. In this case, the regeneration enzyme in Fig. 16.2-2 is identical with the production dehydrogenase. However, conversion rates in this set-up tend to be low because of a given reaction equilibrium, which requires an efficient method to withdraw the products and coproducts.

16.2.2.2.2 Intrasequential Regeneration

One elegant way of *in situ* product removal is to use the product of a first dehydrogenase reaction as substrate for a subsequent enzymatic reaction, thus recycling the oxidized nicotinamide coenzyme (Fig. 16.2-3). Various NAD(P)-dependent enzymes can be applied as regeneration enzymes in this cascade reaction.

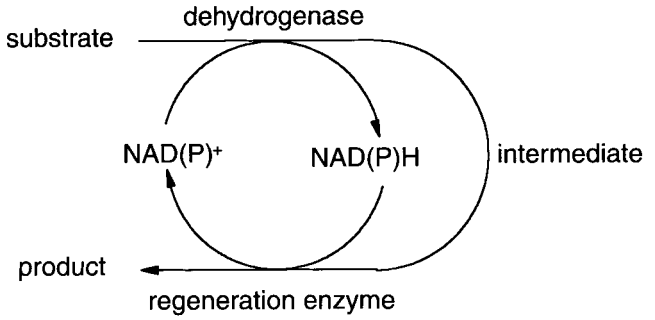


Figure 16.2-3. Intrasequential regeneration of NAD(P)$^+$. The strategy applied is the synthetic coupling of a dehydrogenase-catalyzed oxidation and a regeneration reaction yielding the final product and NAD(P) regeneration.

If the regeneration enzyme is a second dehydrogenase, an overall redoxisomerization takes place. But also monooxygenases are reported as regeneration enzymes thus yielding an overall double oxidation of the substrate (see Sect. 16.2.2.6.1).

16.2.2.3
Molecular Oxygen as Terminal Acceptor

The application of molecular oxygen as oxidant is favorable for several reasons. It is cheap and easily applicable. Furthermore, the high redox potentials of the O_2/H_2O or O_2/H_2O_2 couples (in acidic solution + 1.23 V and + 0.682 V, respectively) result in a strong thermodynamic driving force for the regeneration reaction. Since direct oxidation of NAD(P)H by molecular oxygen is very slow[4], the electron transfer has to be accelerated via enzymatic or chemical techniques.

NADH oxidases (NADH dehydrogenases, E.C. 1.6.99.x) from several organisms have been characterized in recent years[5]. Two types of NADH oxidases can be distinguished, namely those reducing molecular oxygen to water and those performing the reduction to hydrogen peroxide. Interestingly, few examples are found in literature employing NADH oxidases for the regeneration of NAD$^+$, probably because of stability reasons. However, an NADH oxidase from *Thermus aquaticus* was reported to be stable at 80 °C for at least 1 h[6], which might allow small scale applications.

FMN reductase (NAD(P)H dehydrogenase (FMN), E.C. 1.6.8.1) catalyzes the transhydrogenation from NAD(P)H to FMN[7], yielding the oxidized nicotinamide coenzyme and $FMNH_2$, which reacts spontaneously with molecular oxygen (Fig. 16.2-4). The reaction might be coupled to the catalase reaction in order to decrease the degree of enzyme inactivation over longer reaction times. Compared to the non-catalyzed hydride transfer from NAD(P)H to FMN[8], up to 1000-fold increases in the transhydrogenation rate are reported, which is not very high when applied synthetically. In this respect also the operational stability of FMN reductase has to be optimized. Besides the native substrate, cheaper alloxazine-based analogs are also accepted[9].

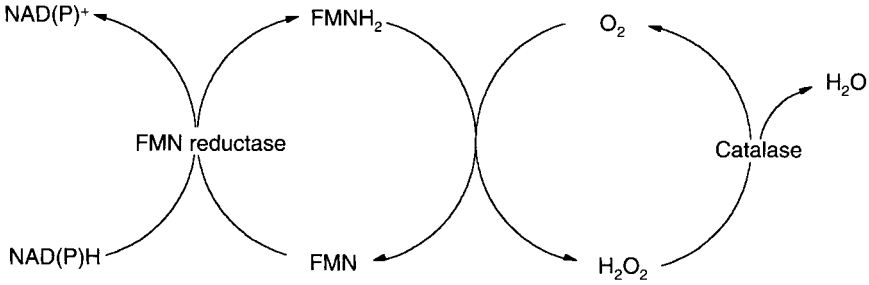


Figure 16.2-4. Transhydrogenation catalyzed by FMN reductase.

Among the chemical mediator systems especially *o-quinones* are capable of accepting the hydride equivalent from reduced nicotinamides. The oxidized mediators are regenerated by molecular oxygen. Since these mediators can also be recycled electrochemically, they are discussed in the following chapter.

16.2.2.4
Electrochemical Regeneration

A very elegant method to regenerate $NAD(P)^+$ from NAD(P)H is to use the anode as terminal electron acceptor. The most common approaches are summarized in Figure 16.2-5.

Direct electrochemical NAD(P)H oxidation (Fig. 16.2-5 A)
The easiest way to oxidize NAD(P)H is to withdraw the excess electrons anodically. Although the formal potential of the $NADH/NAD^+$ couple is – 320 mV [- 324 mV for NADP] vs NHE[10], overpotentials as large as 1 V are required to achieve significant oxidation rates at bare electrodes[11, 12]. The number of enzymes, substrates, and

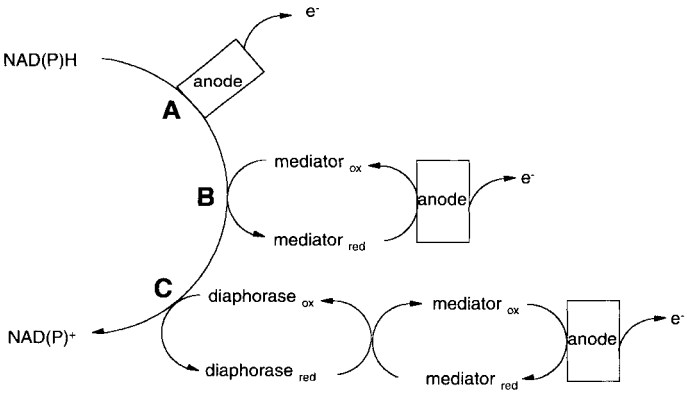


Figure 16.2-5. Electrochemical regeneration of $NAD(P)^+$. A: direct anodic oxidation; B: indirect electrochemical oxidation; C: diaphorase-accelerated indirect electrochemical oxidation.

products that can withstand this oxidizing power is limited. In addition, direct oxidation is often accompanied by electrode fouling, which is attributed to the formation of NAD dimers or stable adducts [12, 13].

Indirect electrochemical NAD(P)H oxidation (Fig. 16.2-5 B,C)
The high overpotentials needed for NAD(P)H oxidation can be considerably lowered by the use of redox mediators. Organic compound (such as *ortho*- and *para*-substituted quinones[14–18], diimines[20], and organic dyes[21–22]) undergoing two-electron transfer processes were found to be ideal fpr $NAD(P)^+$ regeneration. Amongst these, 1,10-phenanthroline-5,6-diones[23, 24] are probably the most potent mediators. Furthermore, quinoid mediators can be generated in the surface of carbon electrodes by oxidative pretreatment[19].

Besides these hydride acceptors, single-electron-transfer mediators (e.g. transition metal complexes[25, 22], viologene derivatives[26, 27], ferrocenes[28], heteropolyanions[29], conducting polymers[30] or ABTS[31] are also capable of oxidizing NAD(P)H. Examples for one- and two-electron acceptors are listed in Table 16.2-2.

These mediators have been applied mostly freely diffusing but also immobilized at the electrode surface. A great variety of immobilization techniques have been used for the preparation of these modified electrodes – the mediator molecules have, for example, been directly adsorbed onto electrode surfaces, incorporated into conducting polymers or covalently linked to functional groups on electrode surfaces.

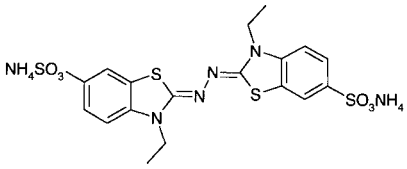
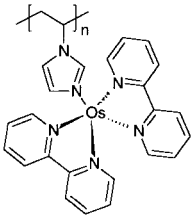
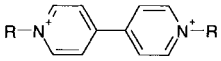
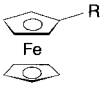
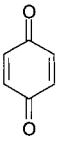
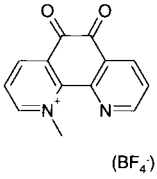
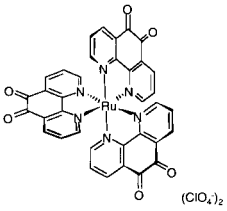
Often the electron transfer between the reduced nicotinamide coenzyme and the mediator is rather slow because of kinetic limitations. In many of these cases electron transfer catalyzed by *diaphorase* (E.C. 1.6.99.x) results in a drastic enhancement of the reaction rate (Fig. 16.2-5 C). Diaphorase-catalyzed $NAD(P)^+$ regeneration was reported for example with methylene blue[32], PQQ[33] (under aerobic conditions), ferrocene[28], *N*-methyl-*p*-aminophenol[34], *N*,*N*-dimethylindoaniline, 2,6-dichlorophenol indophenol (DCIP), $[Fe(CN)_6]^{2-}$[35], viologenes or several quinoid structures[36].

Many of the quinone-based mediators react in their reduced states with molecular oxygen. This aerobic regeneration has the advantage that no additional electrochemical equipment is necessary to perform $NAD(P)^+$ regeneration. On the other hand, reactive oxygen species are generated, which might inactivate enzymes and which therefore need to be removed from the reaction mixture.

It should be mentioned at this point that most of the mediators described here were developed for analytical purposes. Only a few systems were applied to electrochemically driven dehydrogenase-catalyzed oxidations. This is partially because some systems exhibit moderate half-life times.

In conclusion it can be said that, for each individual case, a mediator with a good performance and stability under the given production conditions has to be found.

Table 16.2-2. Selection of frequently used mediators for indirect electrochemical regeneration of $NAD(P)^+$.

One-electron acceptors	
 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid)-diammonium salt (ABTS)	 $[Os(bpy)_2(PVI)_{10}Cl]^+$
 R = Me: methyl viologene R = Bz: benzyl viologene	 Ferrocenes
Two-electron acceptors	
ortho-quinone (and various derivatives)	 *para*-quinone (and various derivatives)
 (BF_4^-) *N*-methyl-1,10-phenanthroline-5,6-dione	 $(ClO_4^-)_2$ $[Ru(PDOn)_3]^{2+}$

16.2.2.5
Photochemical Regeneration

Various methods for photosensitized oxidation of NAD(P)H have been developed[37]. Photochemical methods are based either on the light-induced excitation of a mediator enabling it to oxidize NAD(P)H (reductive quenching mechanism) or on the light-induced excitation of the already reduced mediator, thus facilitating its re-oxidation (oxidative quenching mechanism) (Fig. 16.2-6).

For reductive quenching, photosensitizers such as tin porphyrins[38], methylene blue[39], and other dyes[40] are reported (Fig. 16.2-7). Ruthenium(II) *tris* bipyridine complexes in combination with viologenes are used for oxidative quenching. After

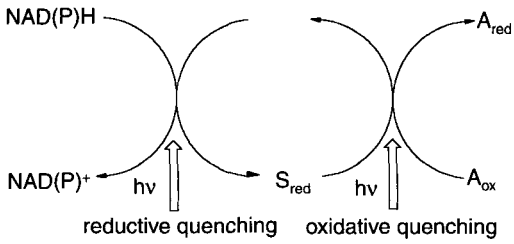
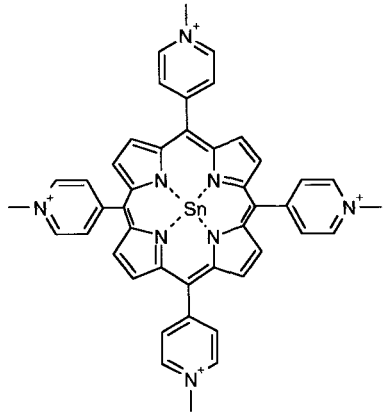
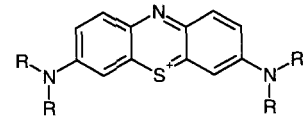


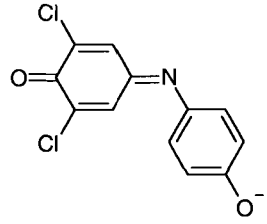
Figure 16.2-6. Electron transfer from NAD(P)H to acceptors (A) via photosensitizers (S) facilitated by photochemical activation.



Sn(II)-meso-tetramethylpyridinium porphyrin



R = CH_3 methylene blue
= H thionine



2,6-dichlorophenol indophenol (DCP/P)

Figure 16.2-7. Photosensitizers used for photochemical regeneration of $NAD(P)^+$ from NAD(P)H.

the oxidation of NAD(P)H, the reduced Ru complex is excited by light. The resulting powerful reduction agent transforms methyl viologene into the radical cation. The electrons from NAD(P)H are usually transferred to molecular oxygen, protons or the anode[38, 40, 41].

Next to soluble photosensitizers, semi-conductors were reported for NAD^+ regeneration[42]. The advantage of these photochemical systems is that some of them utilize visible light, pointing towards the possibility of using sunlight for driving organic reactions. Disadvantageous, however, are the still low performances (TTN and TF of the photosensitizers and coenzymes) and the fact that photoexcitation results in the formation of strong oxidizing agents and the formation of free reactive radicals. Therefore, photochemical regeneration has not become one of the standard procedures, yet[37].

16.2.2.6
Oxidations Catalyzed by Alcohol Dehydrogenase from Horse Liver (HLADH)

HLADH is certainly one of the most prominent and widely used oxidoreductases. The NAD-dependent enzyme is a dimer consisting of two almost identical subunits,

which both contain two zinc atoms[43, 44]. The 3-dimensional structure was elucidated via X-ray analysis[45, 46].

HLADH exhibits a unique combination of a very broad tolerance for primary and secondary alcohols (or aldehydes and ketones in the reductive direction) with an almost invariable and predictable stereospecificity[47, 48]. HLADH exhibits tolerance to many organic solvents[49] and is active even in water-saturated organic solvents[50, 42]. Even though HLADH exhibits a rather poor specific activity in the range of 1–2 U mg^{-1}, it is commercially available at reasonable prices ($ 570/1000 U, Sigma 2001) and, more importantly, is fairly stable even in oxygen-containing media[48]. Also because of that, HLADH has been studied extensively during the last few decades.

16.2.2.6.1 Regeneration of NAD^+ in HLADH-catalyzed Reactions

Various concepts for the enzymatic regeneration of NAD^+ in combination with isolated HLADH have been reported, ranging from a second dehydrogenase such as glutamate dehydrogenase[51, 52] to enzyme-coupled or intrasequential approaches.

A Baeyer-Villiger monooxygenase was applied to oxidize cyclic ketones produced *in situ* by HLADH with concomitant regeneration of NAD^+ (Fig. 16.2-8)[53]. Even though yields and enantiomeric excesses are moderate, this concept has synthetic significance and should be optimized in future.

A very elegant reaction sequence was reported by Tanaka and coworkers[54]. HLADH was used for the kinetic resolution of a series of racemic β-hydroxysilanes yielding one enantiomer in *ee* values ranging from 20 to 97% in reasonable yields and the corresponding β-ketosilane. This β-ketosilane hydrolyzes spontaneously and drives the regeneration of NAD^+ catalyzed by HLADH (Fig. 16.2-9).

Other NAD^+ regeneration approaches are based on the transfer of hydride either to PQQ (catalyzed by diaphorase)[33], directly to flavins[55–57], or to flavins via FMN reductase catalysis[58]. Direct hydride transfer to flavins has the advantage that the alloxazine acceptor can be chosen freely, e. g. cheap riboflavin instead of FAD. On the

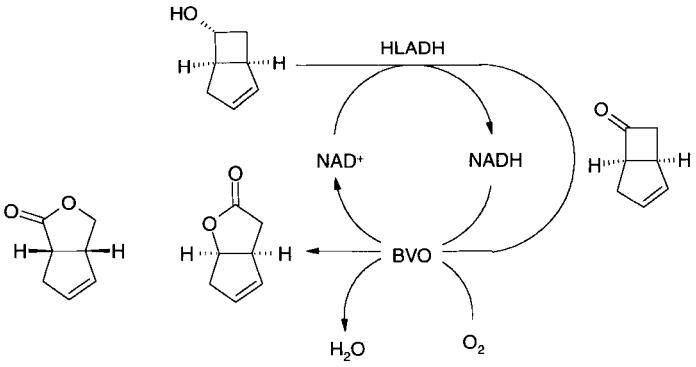


Figure 16.2-8. Intrasequential regeneration of NAD with HLADH and a Baeyer-Villiger monooxygenase (BVO) from *Acinetobacter calcoaceticus*.

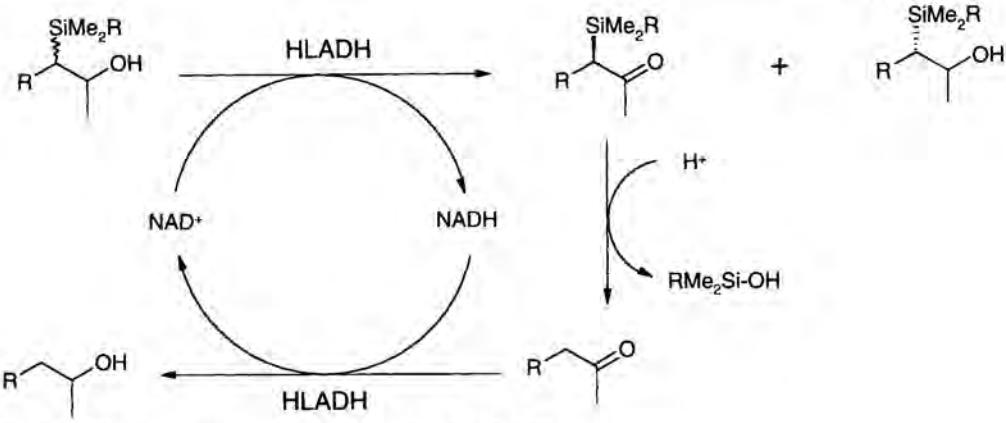


Figure 16.2-9. Intrasequential NAD⁺ regeneration for HLADH-driven kinetic racemate resolution of β-hydroxysilanes.

other hand, the spontaneous hydride transfer suffers from sluggish kinetics ($k = 0.2$ $M^{-1}\,s^{-1}$; turnover rates ranging between 0.06 and 1.8 h^{-1})[59], which is app. 1000-fold slower than the values reported for enzymatic regeneration. For this reason, high excesses of the acceptor have to be applied in order to achieve acceptable regeneration rates. Introduction of FMN reductase accelerates this reaction remarkably.

Electrochemical methods utilizing quinoid mediators[23, 24] or ferrocenes[28] as well as photochemical[42] methods have also been applied to regenerate NAD^+ in combination with HLADH. Especially the electrochemical variants utilizing quinoid shuttle systems proved to be very efficient, with mediator performances as high as 130 catalytic cycles per hour and quantitative yields.

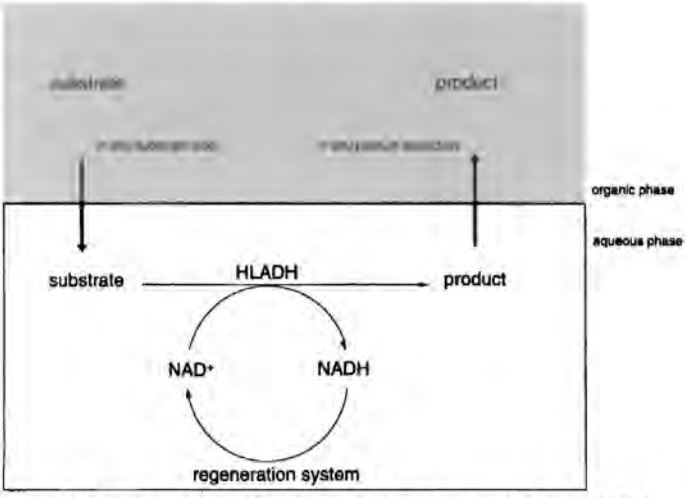
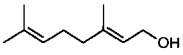
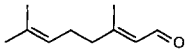
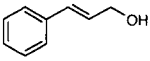
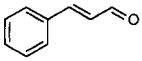
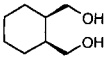
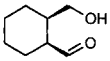
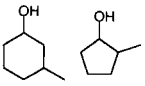
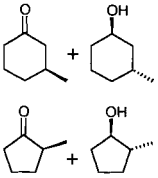
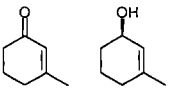


Figure 16.2-10. HLADH-catalyzed oxidations in two-liquid phase systems (in the case of buffer-saturated organic solvents, the aqueous phase is limited to a layer around HLADH).

Table 16.2-3. Synthetic application of HLADH in organic solvents.

Substrate(s)	Product(s)	Solvent	Remarks/Ref.
 Geraniol	 Geranial	Hexane	Plugged-flow reactor for continuous production [2]
 Cinnamylalcohol	 Cinnamylaldehyde	Isopropyl ether	[3]
		Hexane	[4]
 Racemic		Ethyl acetate, chloroform, Isopropyl ether, butyl acetate	HLADH immobilized on glass beads [5]
		Hexane	HLADH in polyacrylamide particles [6]

2 R. Lortie, I. Villaume, M. D. Legoy, D. Thomas, *Biotech. Bioeng.* **1989**, *33*, 229–232.
3 T. Kawamoto, A. Aoki, K. Sonomoto, A. Tanaka, *J. Ferm. Bioeng.* **1989**, *67*, 361–362.
4 J. R. Matos, C.-H. Wong, *J. Org. Chem.* **1986**, *51*, 2388–2389.
5 J. Grundwald, B. Wirz, M. P. Scollar, A. M. Klibanov, *J. Am. Chem. Soc.* **1986**, *108*, 6732–6734.
6 C. Gorrebeck, M. Spanghoe, G. Lanens, G. L. Lemiere, R. A. Dommisse, J. A. Lepoivret, F. C. Adlerweireldt, *Rec. Trav. Chim. Pays-Bas* **1991**, *110*, 231–235.

16.2.2.6.2 HLADH in Organic Media

Several applications of HLADH in organic/aqueous media have been reported (Table 16.2-3). The concept of these two liquid-phase reaction systems is shown schematically in Fig. 16.2-10. This approach is especially suitable for substrates and products with low solubility in aqueous media. Furthermore, the organic phase serves as a sink for products, thus decreasing problems resulting from product inhibition or back reactions.

16.2.2.6.3 Kinetic Resolution of Alcohols using HLADH

Because of its high enantioselectivity, HLADH has found widespread applications in the kinetic resolution of racemic alcohols and α-amino alcohols. Total turnovers of up to 10^8 for HLADH and 800 for NAD were reported with 90% residual activity, yielding the corresponding aldehydes in enantiomeric excesses up to 96%. The α-hydroxy aldehydes were metabolized *in situ* by an aldehyde dehydrogenase to the corresponding α-hydroxy acids (Fig. 16.2-11)[51].

Examples of further kinetic resolutions of racemates via regioselective oxidation using HLADH are given in Fig. 16.2-12.

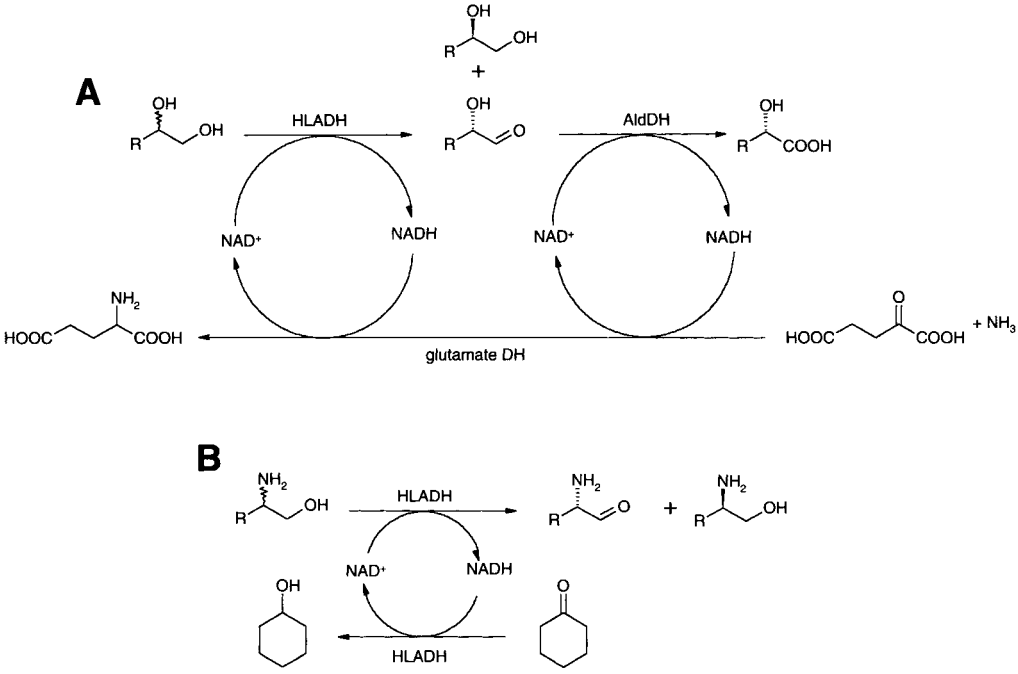


Figure 16.2-11. HLADH as enantioselective catalyst in the kinetic resolution of *vic*-diols (A) and α-amino alcohols (B). R = CH_2OH, CH_2F, CH_2Cl, CH_2Br, CH_3, $CH{=}CH_2$, C_2H_5, CH_2NH_2, $(CH_3)_2$.

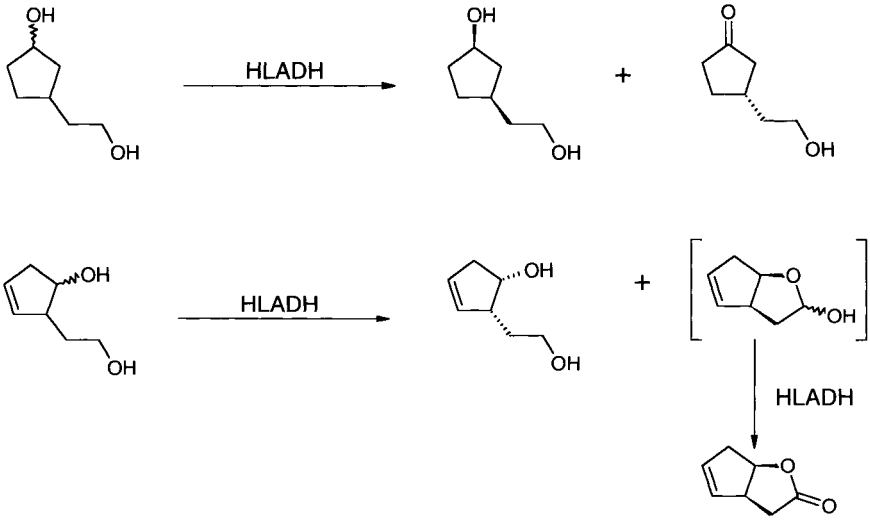


Figure 16.2-12. Chemo- and stereoselective oxidations of *sec*-alcohols.

16.2.2.6.4 HLADH for the Oxidation of *meso*-Compounds

Probably the most prominent application of HLADH is the oxidation of *meso*-diols to homochiral lactones. Both 1,4- and 1,5-diols are accepted as substrates (Table 16.2-4). The overall 4-electron oxidations proceed via two successive steps (tandem oxidation). The enantiomeric excesses often exceed 97 %.

16.2.2.7
Alcohol Dehydrogenase from Yeast (YADH)

Even though the primary sequences differ significantly, YADH exhibits almost the same quaternary structure as HLADH[60]. Nevertheless, far fewer applications in biocatalytic processes are known for YADH than for HLADH. In part this is due to its low overall stability and its low resistance towards organic solvents[61]. Furthermore the substrate spectrum of YADH is limited to primary alcohols and 2-hydroxyalkanes[62]. It has been used in a few oxidative applications[63, 64]. On account of its high specific activity (about 300 U mg^{-1}) together with its very low price (less than 1.2 $/1000 U, Sigma, 2001), YADH has been used as a regeneration enzyme for NADH[65]. In this approach it is a problem that both ethanol and acetaldehyde as cosubstrate and coproduct of the regeneration reaction inactivate YADH and also other enzymes at low concentrations. This problem can be addressed by elegant techniques such as the use of gas membranes. Only volatile compounds such as ethanol or acetaldehyde can pass into the gas phase. This concept has been applied for lactate dehydrogenase (Fig. 16.2-13)[66, 67]. Hazardous acetaldehyde is removed and even recycled to form ethanol by treatment with sodium borohydride in the gas phase. Cycle numbers of over 10 000 are reported.

16.2.2.8
Alcohol Dehydrogenase from *Thermoanaerobium brockii* (TBADH)

TBADH is a NADP-dependent dehydrogenase with remarkable thermostability up to 65 °C[68]. Neither HLADH nor YADH are able to convert linear secondary alco-

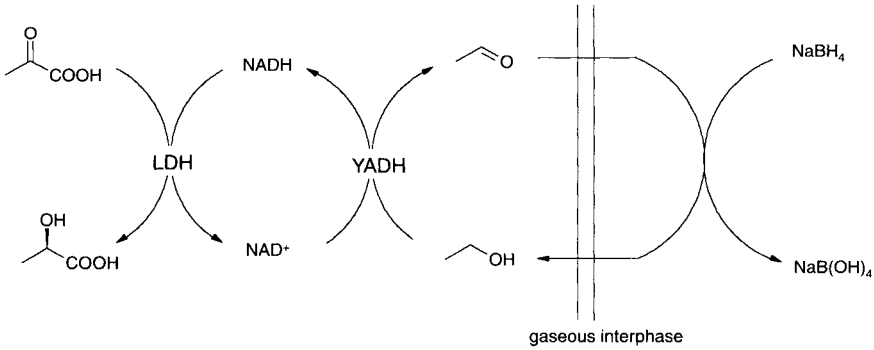
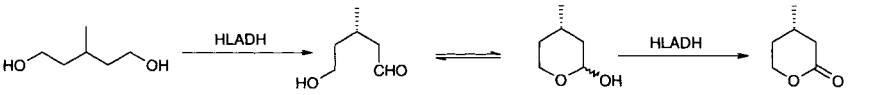
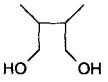
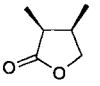
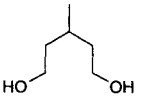
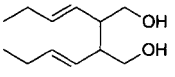
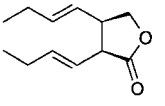
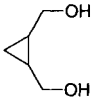
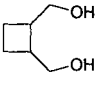
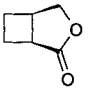
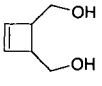
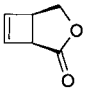
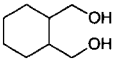
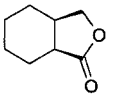
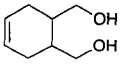
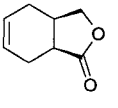
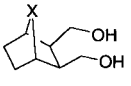
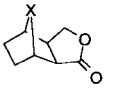
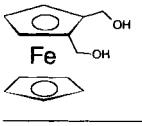
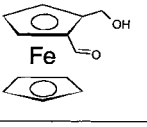


Figure 16.2-13. Regeneration of NADH with YADH. Acetaldehyde diffuses through the gaseous interphase into the second liquid phase where it is regenerated chemically to ethanol.

Table 16.2-4. Examples of HLADH-catalyzed enantioselective oxidations of *meso*-diols.



Meso-diol	Lactone	Yield [%]	*ee* [%]	References
		99	> 97	[7]
		90	95	[8]
		ND[a]	> 97	[9]
		68	> 97	[10]
		90	> 97	[10]
		55	99	[11]
		95	"100"	[8]
		> 99	> 99	[7]
 X = CH_2, O		70	99	[12]
		81	86	[13]

a ND: not determined.

7 G. Hilt, B. Lewall, G. Montero, J. H. P. Utley, E. Steckhan, *Liebigs Ann./Recueil* **1997**, 2289–2296.
8 T. Osa, Y. Kashiwagi, Y. Yanagisawa, *Chem. Lett.* **1994**, 367–370.
9 K. Mori, M. Amaike, J. E. Oliver, *Liebigs Ann. Chem.* **1992**, 1179.
10 Y. Yamazaki, K. Hosono, *Tetrahedron Lett.* **1989**, *30*, 5313–5314.
11 M.-E. Gourdel-Martin, C. Comoy, F. Huet, *Tetrahedron: Asym.* **1999**, *10*, 403–404.
12 R. N. Patel, M. Liu, A. Banerjee, S. L., *Ind. J. Chem.* **1992**, *31B*, 832–836.
13 Y. Yamazaki, K. Hosono, *Tetrahedron Lett.* **1988**, *29*, 5769–5770.

hols. TBADH fills this gap: its activity is highest for secondary alcohols, being low for primary alcohols[48]. Because of this rather narrow substrate spectrum, TBADH is mostly used for the regeneration of NADPH. Only a few synthetic applications are reported[24, 69]. Figure 16.2-14 gives one example where YADH was used simultaneously as an oxidizing enzyme and a NADPH regeneration enzyme (intrasequential cofactor regeneration).

16.2.2.9
Glycerol Dehydrogenase (GDH, E.C. 1.1.1.6)

GDH was isolated from various bacterial strains, especially from *Schizosaccharomyces pombe*[70, 71] and *Cellulomonas* sp.[72, 73].

It displays a somewhat complementary substrate specificity to HLADH. While HLADH oxidizes *meso*-diols with secondary hydroxyl groups rather badly, they are readily oxidized by GDH to the corresponding (*S*)-α-hydroxyketones[1]. Furthermore, the natural substrate glycerol is transformed to achiral dihydroxy acetone by GDH while HLADH produces optically active (*S*)-glyceraldehyde. In many cases GDH seems to prefer secondary hydroxyl groups (Table 16.2-5), although this rule of thumb has some exceptions.

In aqueous buffers GDH exhibits only low enantioselectivity, e.g. for the kinetic resolution of 1-phenyl-1,2-ethanediol (which is most probably due to spontaneous racemization via enolization)[74]; furthermore, it suffers from pronounced product inhibition, accounting for low yields. Both problems (product inhibition and

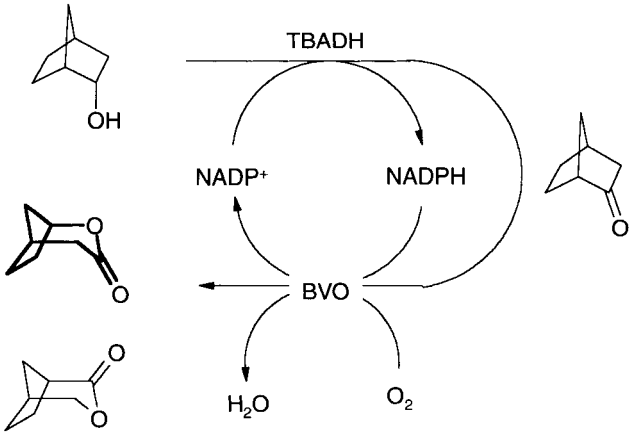
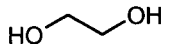
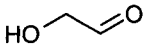
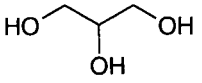
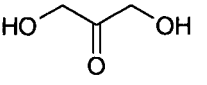
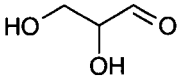
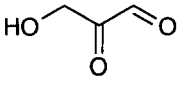
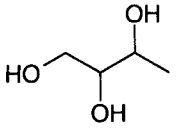
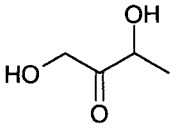
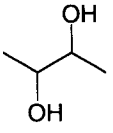
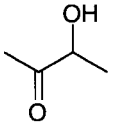
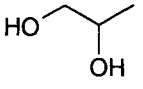
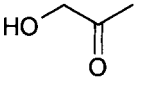


Figure 16.2-14. Intrasequential regeneration of NADP with TBADH and a Baeyer-Villiger monooxygenase (BVO) from *Acinetobacter calcoaceticus*.

Table 16.2-5. Alcohol oxidations catalyzed by glycerol dehydrogenase[14].

Substrate		Product
	→	
	→	
	→	
	→	
	→	
	→	

14 J. H. Marshall, J. W. May, J. Sloan, *J. Gen. Microbiol.* **1985**, *131*, 1581–1588.

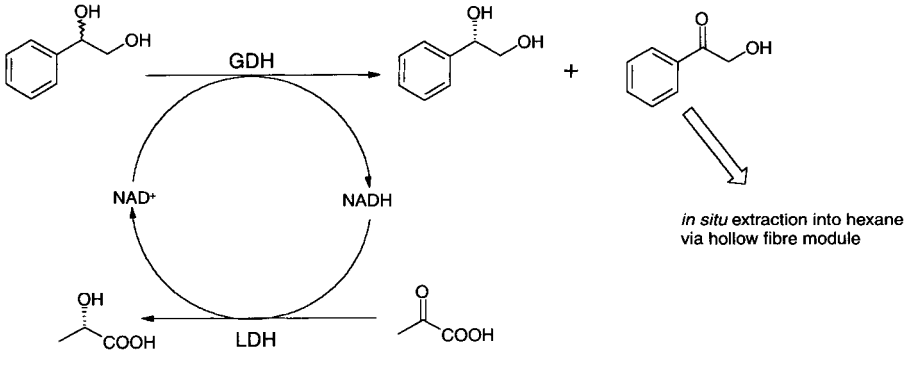


Figure 16.2-15. Deracemization of *rac* 1-phenyl-1,2-ethandiol coupled to *in situ* product extraction via a hollow fiber module.

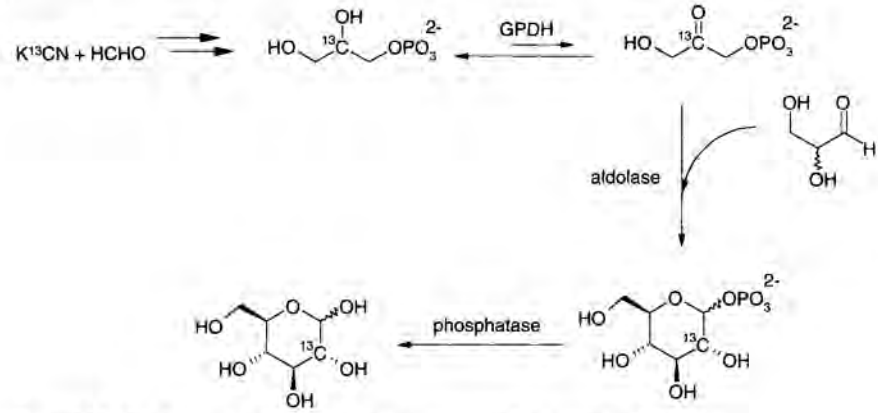


Figure 16.2-16. Synthesis of ^{13}C-labeled sugars in a tandem reaction of GPDH, aldolase, and phosphatase.

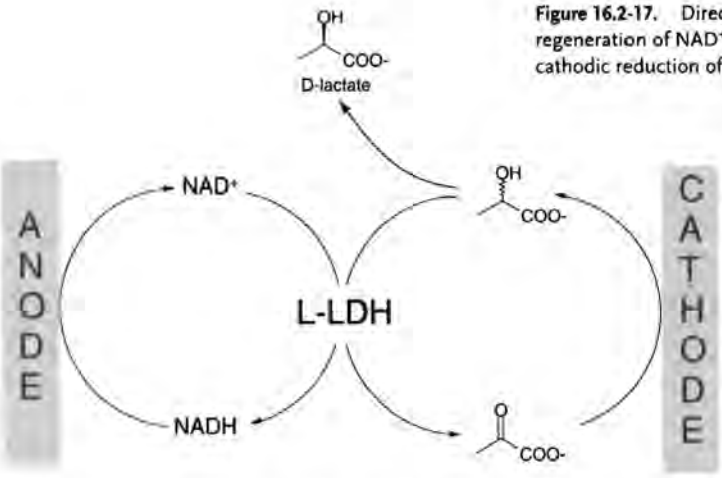


Figure 16.2-17. Direct anodic regeneration of NAD$^+$ coupled to cathodic reduction of pyruvate.

racemization) can be solved by *in situ* extraction into a second (organic) phase (Fig. 16.2-15)[74].

This biphasic system yielded higher *ee* values (99% instead of 58%) at maximal theoretical conversions (50% instead of 38%) in significantly shorter reaction times (60 h instead of 170 h) compared to the solely aqueous system.

Since GDH contains autooxidizable thiol groups, it is necessary to perform such reactions in media essentially free from oxygen.

16.2.2.10

Glycerol-3-phosphate Dehydrogenase (GPDH, E.C. 1.1.1.8)

GPDH has been isolated from various organisms. The enzyme from rabbit muscle is commercially available. Its synthetic applications are limited because of its very

narrow substrate spectrum it almost exclusively accepts L-glycerol-3-phosphate[75, 76]. The product 3-hydroxyacetone phosphate, however, is an essential substrate of aldolases and therefore can serve as a building block in the enzymatic synthesis of non-native sugars and polyols. Although the redox equilibrium of GPDH favors the reduced substrates even more than in the case of GDH, it has been employed in the synthesis of radioactively labeled carbohydrates starting from $K^{13}CN$ and formaldehyde (Fig. 16.2-16)[77, 78]. Depending on the substrates, single- or double-labeled glucose, fructose or sorbose are available by the sequence outlined in Fig. 16.2-16.

16.2.2.11
Lactate Dehydrogenase (LDH, E.C. 1.1.1.27)

LDH was used to catalyze the deracemization of lactate in a very elegant electrochemical approach. The driving force of the endergonic reaction was supplied by anodic regeneration of NAD^+ and cathodic reduction of pyruvate (Fig. 16.2-17)[79, 80]. Thus, both LDH products were removed efficiently, avoiding product inhibition. The electrochemical reduction of pyruvate leads to racemic lactate, producing 50% of the desired product and 50% of "new" substrate for LDH. An

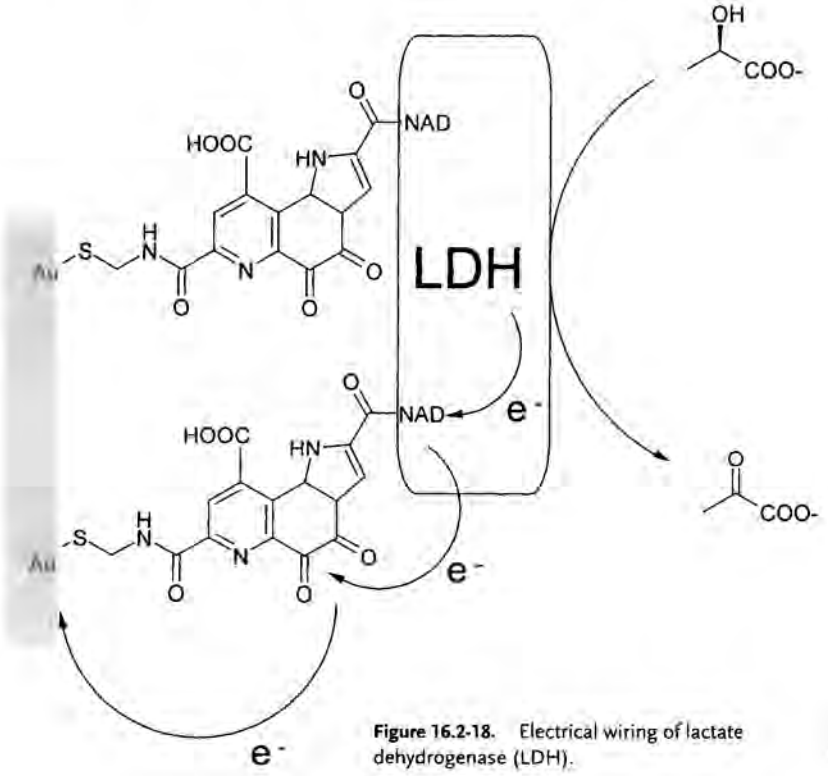


Figure 16.2-18. Electrical wiring of lactate dehydrogenase (LDH).

interesting approach to direct "electrical wiring" of LDH to an electrode was reported recently (Fig. 16.2-18)[81].

NAD was covalently linked via a PQQ spacer to a gold electrode. This modified electrode is capable of binding LDH over the exposed nicotinamide groups. Upon oxidation of lactate to pyruvate the excess electrons tunnel from NADH in the active site to PQQ and eventually to the anode. Thus, a kind of electrical linkage between the enzyme and the electrode is established. The enzymes were crosslinked, as LDH is a homotetramer and might dissociate during the reaction. This approach is not only useful for electrochemical biosensors but might be transferred to other oxidoreductase reactions.

16.2.2.12
Carbohydrate Dehydrogenases

Many so-called polyol dehydrogenases have been reported in literature, for example various glucose dehydrogenases, mannitol dehydrogenase, fructose dehydrogenase, and uridine-5'-diphosphoglucose dehydrogenase. Glucose dehydrogenase (E.C. 1.1.1.47) was applied for the production of D-gluconic acid in a plug-flow reactor with direct electrochemical regeneration of NAD^+[82]. Glucose-6-phosphate dehydrogenase (E.C. 1.1.1.49) is a common regeneration enzyme for NADPH[69]. Most polyol dehydrogenases are not specific for their native substrate, but also catalyze the oxidoreduction of various carbohydrates. Thus, they can be applied for the production of (non-)natural sugars which are especially valuable in the sweetener industry. Yet their applications are limited compared to the polyol oxidases (see Sect. 16.2.3)

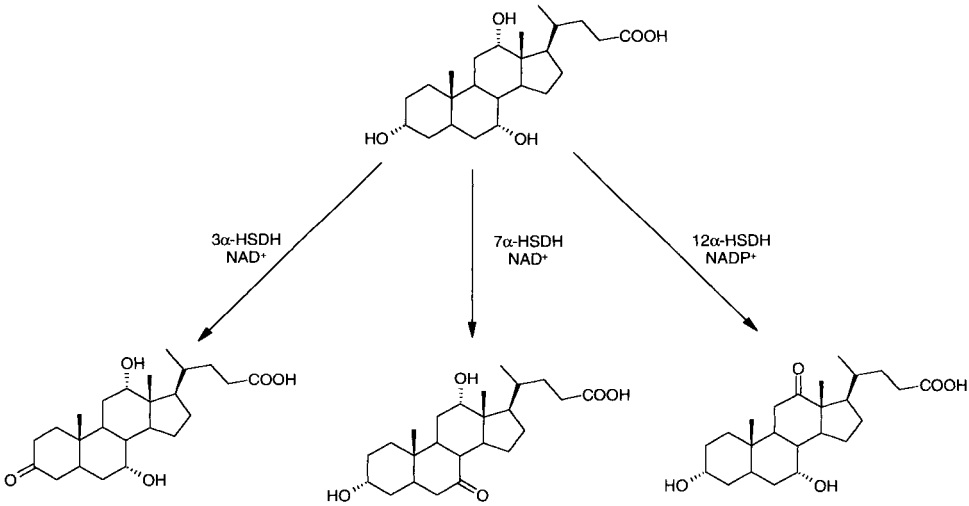


Figure 16.2-19. Regioselective oxidation of cholic acid by hydroxysteroid dehydrogenases.

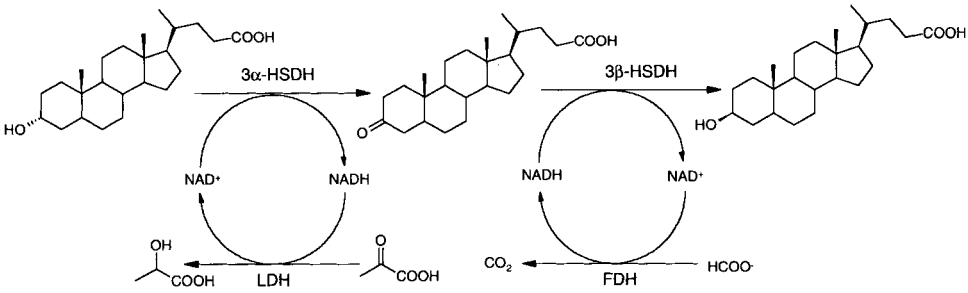


Figure 16.2-20. 3α- and 3β-hydroxysteroid dehydrogenase (HSDH) catalyzed stereoinversion in steroids.

16.2.2.13
Hydroxysteroid Dehydrogenases (HSDH)

The hydroxysteroid dehydrogenases comprise another group of synthetically interesting dehydrogenases. For many hydroxylated positions of the steroid backbone, individual NAD(P)$^+$ dependent dehydrogenases exist, which selectively oxidize the respective residue.

For example, the three hydroxy groups of cholic acid in the 3-,7-, and 12-positions can all be oxidized regioselectively (Fig. 16.2-19)[83–85].

In addition to the regioselective oxidation of the hydroxy groups in virtually every position, a discrimination of the absolute stereochemistry can be achieved by various α- or β-selective HSDHs. Thus, the stereoinversion of various steroids was achieved by successive oxidation at position 3 with 3α-HSDH and subsequent reduction with 3β-HSDH (Fig. 16.2-20)[84]. Hydroxy functions in other positions were not modified, and the products at the end of the sequence were essentially pure. Because of the low solubility of the reactants, biphasic systems with ethyl (butyl) acetate as organic solvents were used as reaction media.

16.2.2.14
Other Dehydrogenases

In addition to the alcohol dehydrogenases mentioned above, ADHs from various other sources were examined, especially with respect to increased stability, resistance to organic solvents, and catalytic properties.

A NAD$^+$ dependent ADH isolated from *Sulfolobus solfataricus* was found to exhibit better thermostability than HLADH [$t^1/_2$ (60 °C) = 20 h] together with a distinctive preference for (*S*)-alcohols (complementary to HLADH)[86]. The enzyme has a broad substrate specificity that includes linear and branched primary alcohols and linear and cyclic secondary alcohols[48]. The highly purified enzyme exhibits a specific activity of 4 U mg^{-1} (for benzyl alcohol at 65 °C)[87, 88]. To date, this enzyme is not commercially available.

Hummel *et al.* established a new route to enantiomerically pure alcohols by the

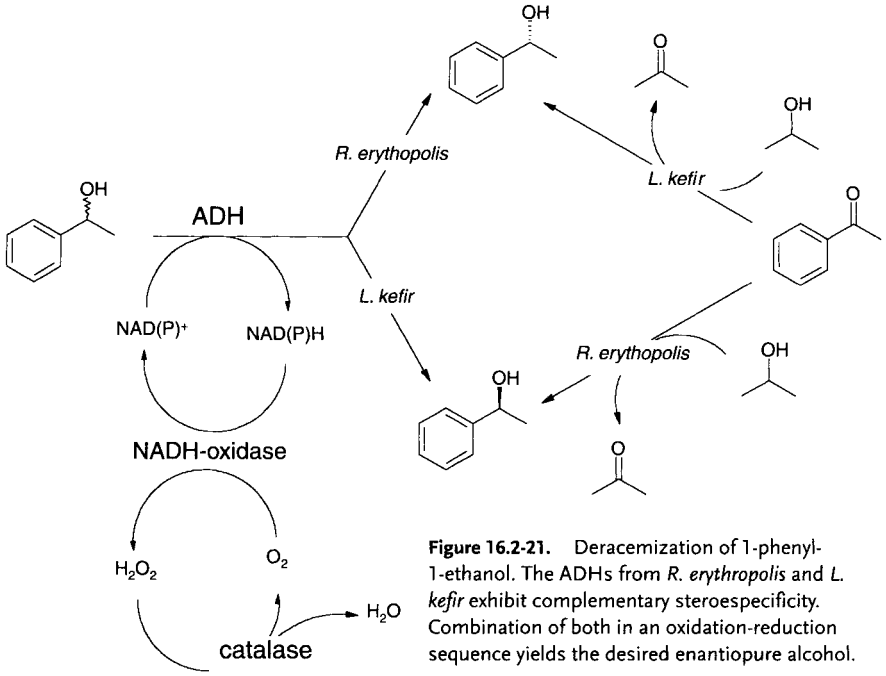


Figure 16.2-21. Deracemization of 1-phenyl-1-ethanol. The ADHs from *R. erythropolis* and *L. kefir* exhibit complementary steroespecificity. Combination of both in an oxidation-reduction sequence yields the desired enantiopure alcohol.

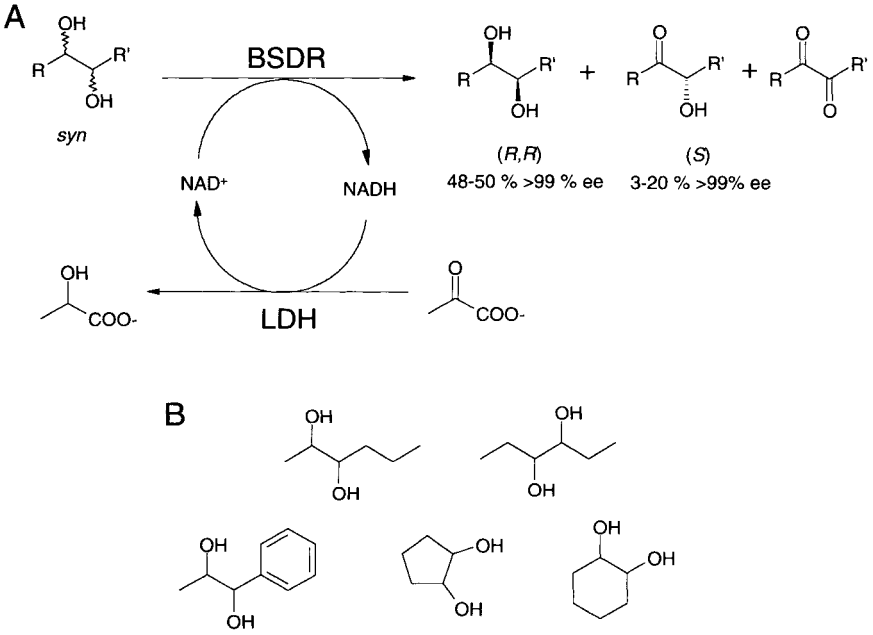


Figure 16.2-22. Kinetic resolution of racemic *syn*-diols by *Bacillus stearothermophilus* diacetyl reductase (BSDR). A: reaction with LDH-catalyzed regeneration of NAD^+; B: selection of *syn*-diols applied.

combination of a (*R*)-specific, NADP-dependent ADH from *Lactobacillus kefir* and a (*S*)-specific, NAD-dependent ADH from *Rhodococcus erythropolis*[89]. In a first step, a kinetic resolution yielded 50% of the desired alcohol. Subsequently the ketone was reduced with the suitable ADH, finally yielding the desired optically pure enantiomer in 100% yield (Fig. 16.2-21).

Recently, diacetyl reductase (Acetoin reductase, E.C. 1.1.1.5) from *Bacillus stearothermophilus* (BSDR) was reported to be a powerful catalyst in the oxidative kinetic resolution of *vic*-diols (Fig. 16.2-22)[90]. All *syn*-diols tested yielded the enantiopure (*R,R*) diols in almost maximum theoretical yields, α-hydroxy ketones were largely further oxidized to the corresponding diketones. Oxidation of *vic-anti* diols only gave *ee* values in the range of 62–76%.

16.2.3
Oxidases as Catalysts

16.2.3.1
General Remarks

Oxidases utilize molecular oxygen as terminal electron acceptor. This can be considered as aerobic regeneration of the prosthetic group of the oxidase. At first glance, this seems to offer a simpler enzymatic oxidation procedure compared to the coenzyme-dependent dehydrogenases or monooxygenases. However, with few exceptions such as cytochrome c oxidase[91], some NADH oxidases[92] or laccases[93], which reduce molecular oxygen directly to water in an overall four-electron transfer step, O_2 reduction generally leads to hydrogen peroxide (transfer of two electrons) or to the superoxide radical anion (transfer of one electron) as primary reduction products.

16.2.3.2
Methods to Diminish/Avoid H_2O_2 formation

Autoregeneration of oxidases with concomitant catalase-catalyzed disproportionation of hydrogen peroxide is a simple and effective regeneration method (Fig. 16.2-23); it is quite commonly used with oxidase reactions.

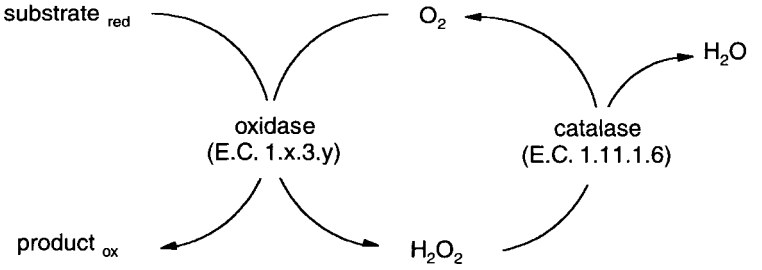


Figure 16.2-23. Coupling of oxidase autoregeneration and catalase for dismutation of hydrogen peroxide.

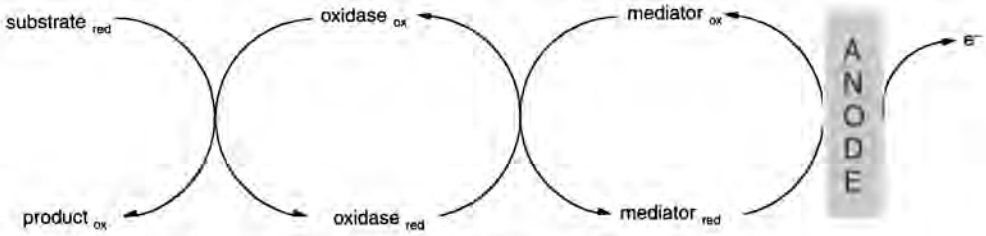


Figure 16.2-24. Indirect electrochemical regeneration of an oxidase.

Hydrogen peroxide, however, is highly reactive and irreversibly inhibits enzyme activity (also catalase) even in low concentrations.

Hydrogen peroxide can be avoided if excess electrons are transferred to the anode. However, direct electron transfer between enzymes and solid electrodes is usually very slow because the enzymatic active sites are often deeply buried within the protein shell and therefore inaccessible for the electrode (the tunneling probability of electrons is a function of distance). In order to accelerate the electron transfer, low molecular weight redox active substances can be used to shuttle the electrons between the enzyme and the electrode. This indirect electrochemical enzyme regeneration is represented schematically in Figure 16.2-24.

For the anaerobic electrochemical regeneration of a given oxidase, a suitable mediator can be chosen from various organometallic complexes, especially ferrocenes[94–102], but also bipyridine/phenanthroline, terpyridine, or hexacyano complexes[103, 104]. Also, quinoid salts such as TTF/TCNQ (tetrathiofulvalene/tetracyanoquinodimethane)[105, 106] as well as benzoquinones[107] and redox dyes such as phenazine and phenothiazine derivatives (MPMS, thionin, azure A, and azure C)[108] proved to be useful redox agents for indirect electron transfer. Even incorporation of oxidases into conducting polymers made of polypyrrole or polythiophene derivatives proved to function for electrochemical regeneration[109].

It should be mentioned at this point that most of the research in the field of electrochemical oxidase regeneration concentrates on analytical applications, inspired by the search for electrochemical biosensors[110].

However, it was demonstrated that indirect electrochemical methods are suitable for prolonging oxidase operational stability. In a particular example, glucose oxidase (E.C. 1.1.3.4) was immobilized on a carbon felt anode and regenerated with the benzoquinone/hydroquinone redox couple (Fig. 16.2-25)[107]. Thus, the operational stability of glucose oxidase could be increased at least 50 times compared to the use of molecular oxygen as oxidant. Productivities as high as 100 g h^{-1} L^{-1} were reached.

One disadvantage of the electrochemical methods is the need for rather elaborate equipment. Recently, Baminger *et al.* proposed a novel concept of enzymatic regeneration of a range of redox mediators including quinones and various redox dyes[93]. Instead of reoxidizing these mediators via the anode, laccases are employed. Laccases (E.C. 1.10.3.2) are multi-copper oxidases[111] that are found in various trees and fungi[112, 113]. Laccases catalyze the oxidation of various structurally diverse

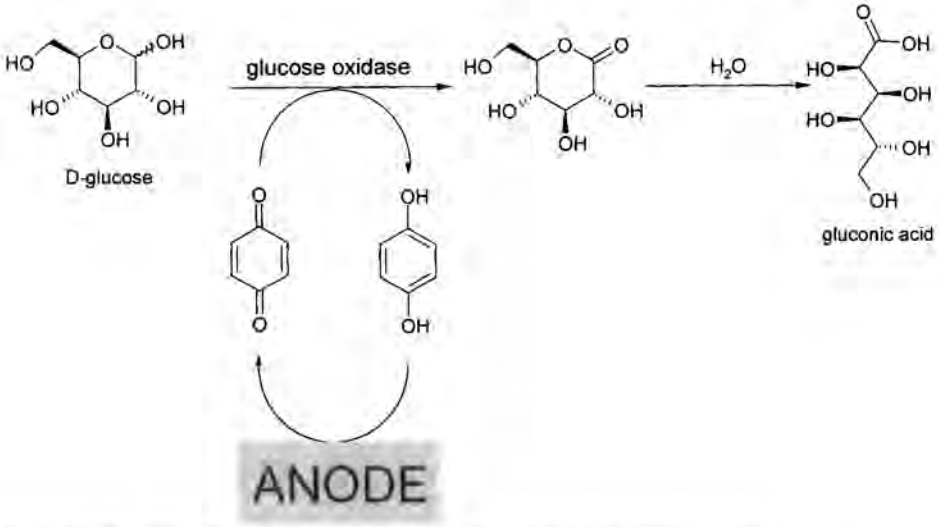


Figure 16.2-25. Indirect electro-enzymatic oxidation of glucose using glucose oxidase.

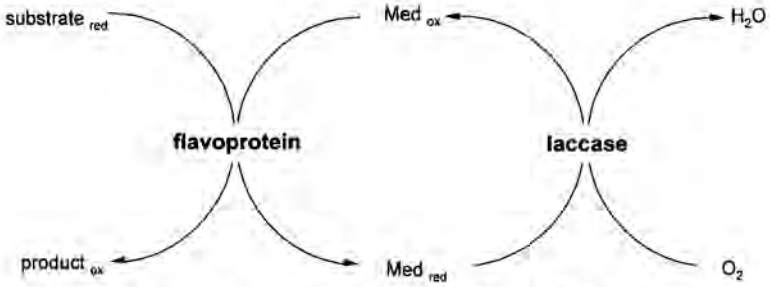


Figure 16.2-26. Laccase-based regeneration concept for oxidized flavoproteins (oxidases).

substances with concomitant reduction of molecular oxygen to water[114], thus avoiding the generation of hazardous hydrogen peroxide (Fig. 16.2-26).

This regeneration concept was tested with pyranose oxidase (P2O, E.C. 1.1.3.10)[93]. Interestingly, it was found that P2O shows higher affinity for some mediators than for O_2 (K_M value for 1,4-benzoquinone is 120 mM compared to 650 mM for O_2) with otherwise comparable activities yielding a 6 times higher k_{cat}/K_M value. Preparative scale biotransformations could be performed with two-fold volumetric productivities. The TTNs were 1.1×10^6 for P2O, with a residual activity of 85%, and 800 for 1,4-benzoquinone. Similar results were obtained with the enzyme cellobiose-dehydrogenase (E.C. 1.1.99.18), which is incapable of autoregeneration, in combination with ABTS or DCIP and laccase.

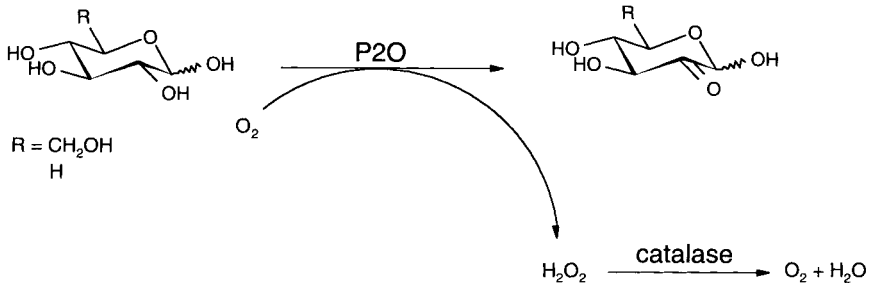


Figure 16.2-27. Oxidation of carbohydrates specifically at C-2 by pyranose oxidase (P2O).

16.2.3.3

Pyranose Oxidase (P2O, E.C. 1.1.3.10)

P2O is common among wood-degrading basidiomycetes[115]. It has been isolated and characterized from various microorganisms[116]. Although the substrate specificity varies to some extent among the P2Os isolated from different fungi, P2Os have some properties in common, such as the homotetrameric structure with covalently bound FAD. The main metabolic role of P2O appears to be as a constituent of the fungal ligninolytic system that provides the lignin-degrading lignin peroxidase and manganese peroxidase with hydrogen peroxide[117].

Natural substrates of P2O are probably D-glucose, D-galactose, and D-xylose, which are abundant in lignocellulose and which are oxidized to the corresponding 2-keto sugars. In addition, P2O exhibits significant activity with a number of other carbohydrates[118]. During such oxidations, electrons are transferred to molecular oxygen, yielding hydrogen peroxide. In addition, benzoquinones, 2,6-dichloroindophenol, as well as ABTS were reported to function as electron acceptors[93, 119]. Interestingly, up to 11-fold increased reactivity (compared to molecular oxygen as electron acceptor) was found.

P2O is currently used in various analytical applications, e.g., in clinical chemistry for the determination of 1,5-anhydro-D-glucitol, an important marker for glycemic control in diabetes patients[116], or in amperometric biosensors for the detection of monosaccharides[120, 121]. For the last two decades, P2O has received increased attention as the key catalyst in several biotechnological applications. Only a few can be mentioned here.

The essential structural requirements of substrates for P2O are the six-membered ring of pyranoid saccharides and an equatorially orientated 2-OH group[122]. In some cases regioselective oxidation at C-3 was observed[118]. The general reaction scheme is given in Fig. 16.2-27. Table 16.2-6 gives a selection of preparative oxidations reported with P2O.

The "Cetus process"

P2O is involved in the so-called "Cetus process", in which D-fructose is produced from cheap D-glucose (Fig. 16.2-28).

Table 16.2-6. Substrates and oxidation products of pyranose oxidase.

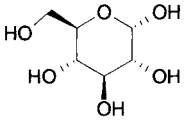
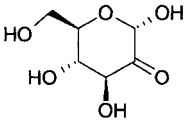
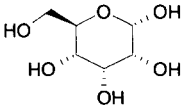
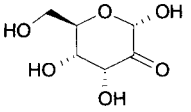
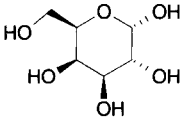
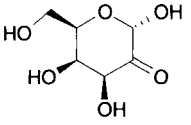
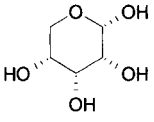
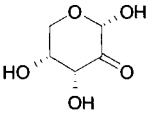
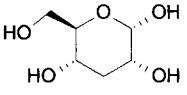
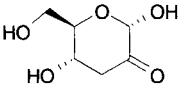
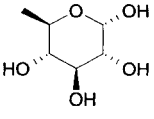
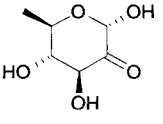
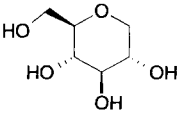
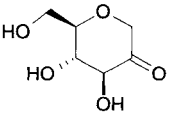
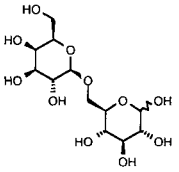
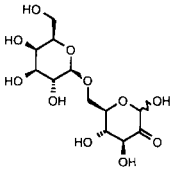
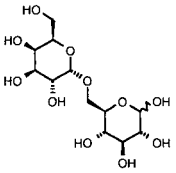
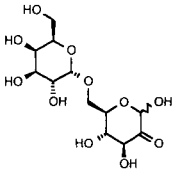
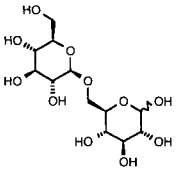
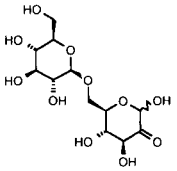
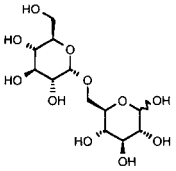
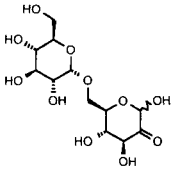
Substrate	Product	Yield [%][a]	Activity [%][a]	References
	Oxidation of monosaccharides			
 D-Glucose	 D-Glucosone	100	100	[15]
 D-Allose	 D-Ribo-hexos-2-ulose	94	40	[15]
 D-Galactose	 D-Lyxo-hexos-2-ulose	70	8	[15]
 D-Ribose	 D-Erythro-pentos-2-ulose	5	very low	[15]
 3d-D-Glucose	 3d-D-Erythro-hexos-2-ulose	100	96	[15]
 6d-D-Glucose	 6d-D-Ribo-hexos-2-ulose	100	92	[15]
 1,5-Anhydro-D-glucitol	 1,5-Anhydro-D-fructose	100	75	[15]

Table 16.2-6. (cont.).

Substrate	Product	Yield [%][a]	Activity [%][a]	References
	Oxidation of Disaccharides			
 Allolactose	 Allolactulose	100	ND[b]	[16]
 Meliobiose	 Meliobiulose	100	ND[b]	[16]
 Gentiobiose	 Gentiobiulose	100	ND[b]	[16]
 Isomaltose	 Palatinose	100	ND[b]	[16]

a expressed as percentage of yield and activity of D-glucose oxidation; b ND: not determined.

15 S. Freimund, A. Huwig, F. Giffhorn, S. Köpper, *Chem. Eur. J.* **1998**, *4*, 2442–2455.

16 C. Leitner, P. Mayr, S. Riva, J. Volc, K. D. Kulbe, B. Nidetzky, D. Haltrich, *J. Mol. Cat. B: Enzymatic* **2001**, *11*, 407–414.

Hydrogen peroxide is not merely dismutated by catalase, but used as substrate in a second enzyme cascade reaction producing propylene oxide[123–125]. In an alternative process[126] the reduction step was performed enzymatically using aldose reductase and formate dehydrogenase for NADH regeneration. Thus, essentially glucose free D-fructose was obtained.

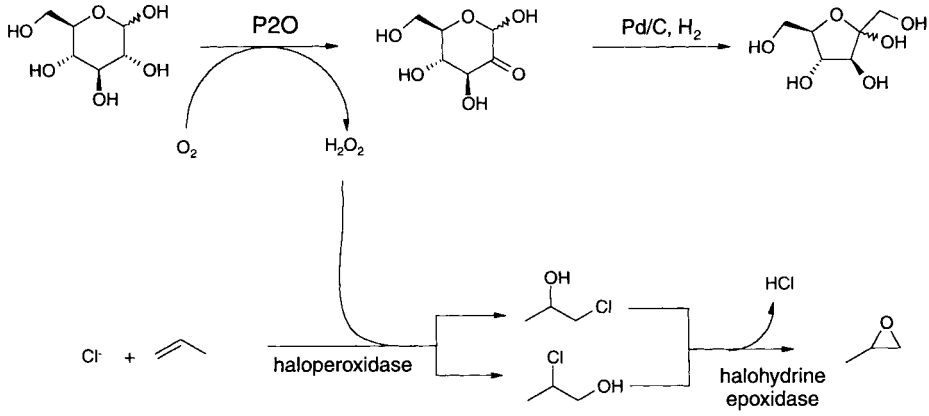
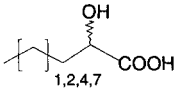
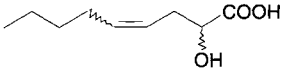
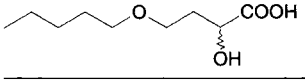


Figure 16.2-28. Isomerization of D-glucose to D-fructose with pyranose oxidase (P2O) and coupling of hydrogen peroxide to a synthetic reaction (Cetus process).

Table 16.2-7. Kinetic resolution of some racemic 2-hydroxy acids to the (*R*)-2-hydroxy acids and the corresponding 2-keto acids[17].

Substrate	Yield [%]	*ee* [%]
	49–50	> 98
	50	> 99
cis/trans		
	47	86

17 W. Adam, M. Lazarus, B. Boss, C. R. Saha-Möller, H.-U. Humpf, P. Schreier, *J. Org. Chem.* **1997**, *62*, 7841–7843.

16.2.3.4
Glycolate Oxidase (E.C. 1.1.3.15)

Glycolate oxidase is a peroxisomal enzyme that is found in the leaves of many green plants and in the liver of mammalians. The enzyme isolated and for economic reasons only partially purified from spinach (*Spinacia oleracea*) was applied to the enantioselective oxidation of various 2-hydroxy acids yielding the corresponding 2-keto acid and the remaining (*R*) alcohol[127]. Enantiopure 2-hydroxy acids are valuable building blocks in the synthesis of glycols[128], haloesters[129] or epoxides[130]. Unless the steric demand of the substituents close to the alcohol function is too big, the oxidation proceeds smoothly to the full theoretical conversion with enantiomeric excesses of the alcohols usually in the range of 98–99% (Table 16.2-7).

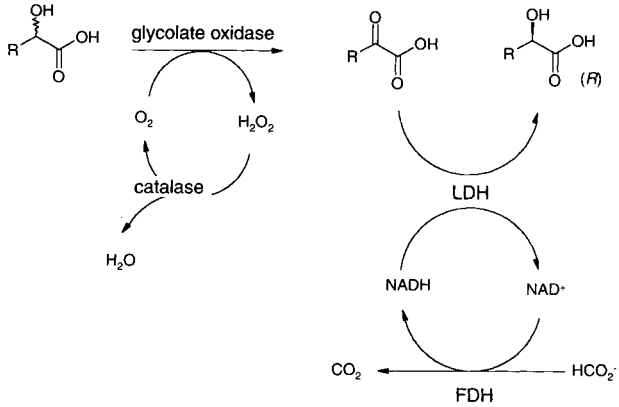
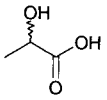
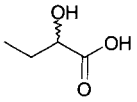


Figure 16.2-29. Deracemization of racemic 2-hydroxy acids in a combination of glycolate oxidase and lactate dehydrogenase (LDH).

Table 16.2-8. Conversion of racemic 2-hydroxy acids into (*R*)-2-hydroxy acids by the combined action of glycolate oxidase and D-lactate dehydrogenase[18].

Substrate	Oxidase [U]	Dehydrogenase [U]	Reaction time [h]	Yield [%]	*ee* [%]
	2	450	66	100	> 99
	2	900	210	100	94

One unit (U) is defined as the amount of enzyme which converts 1 μmol of substrate per minute.

18 W. Adam, M. Lazarus, C. R. Saha-Möller, P. Schreier, *Tetrahedron Asymmetry* **1998**, 9, 351–355.

Kinetic resolutions have a maximum yield of only 50%. Therefore, a second enzymatic process was added after completion of the glycolate oxidase-catalyzed kinetic resolution[131]. By addition of D-lactate dehydrogenase (E.C. 1.1.1.28) together with formate dehydrogenase for NADH regeneration, enantiospecific reduction of the 2-keto acid was achieved. Overall, a quantitative transformation (deracemization) of the racemic 2-hydroxy acid into the corresponding (*R*)-2-hydroxy acid was achieved (Fig. 16.2-29).

Unfortunately, this process cannot be performed in a more elegant and more efficient one-pot synthesis. On the one hand, the pH optima for the three enzymes are not compatible with each other, and on the other, lactate dehydrogenase is air sensitive. In addition to this, glycolate oxidase also catalyzes the reverse reaction under aerobic conditions, thus lowering the *ee*-value. Therefore, the reaction mixture is filtered (glycolate oxidase can be reused) and, after pH adjustment, the second enzymatic transformation is performed. Table 16.2-8 shows some results of this procedure.

Glycolate oxidase has been studied thoroughly not only for specific oxidation of

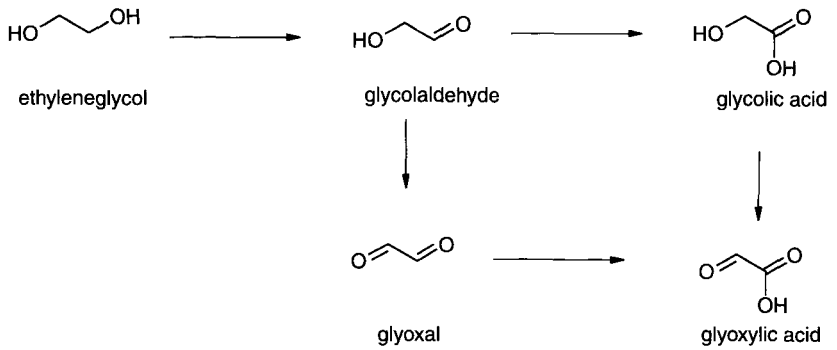


Figure 16.2-30. Sequential oxidation of ethylene glycol to glycolic acid.

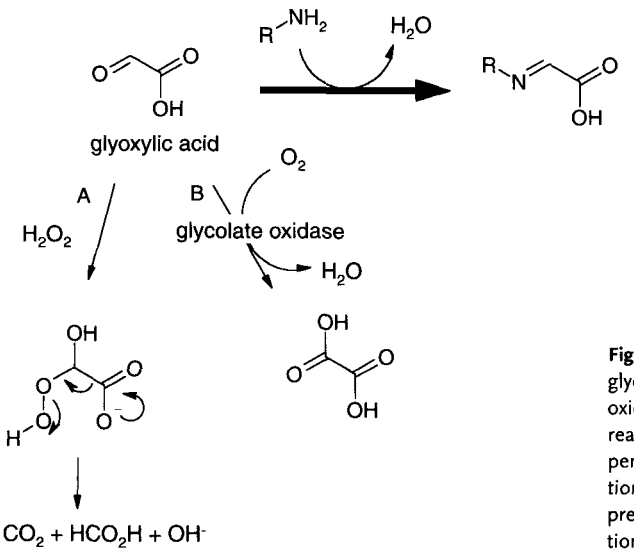


Figure 16.2-31. Synthesis of glyoxylic acid by glycolate oxidase. The undesired side-reactions (A) with hydrogen peroxide and (B) overoxidation by glycolate oxidase are prevented by *in situ* formation of an imine.

(*S*)-2-hydroxy propionic acid (lactate)[132] and for the kinetic resolution of racemic 2-hydroxy acids[127, 131], but also for selective oxidations of 1,2-diols such as ethylene glycol (Fig. 16.2-30).

Reports on the specific conversion of glycolic acid into glyoxylic acid are numerous. Isobe *et al.* Introduced an *in vivo* system utilizing *Alcaligenes* sp. isolated from media containing 1,2-propanediol. By carefully adjusting the pH, a yield of 95 % was obtained[133].

DiCosimo and coworkers optimized the *in vitro* production of glyoxylic acid from glycolic acid with glycolate oxidase from spinach[134]. Improvements in operational stability as well as in productivity were achieved by enzyme immobilization either onto a solid matrix[135] or in permeabilized, metabolically inactive cells of *Pichia pastoris* or *Hansenula polymorpha*, containing overexpressed glycolate oxidase from spinach together with catalase. The undesired oxidation of glyoxylic acid by hydrogen

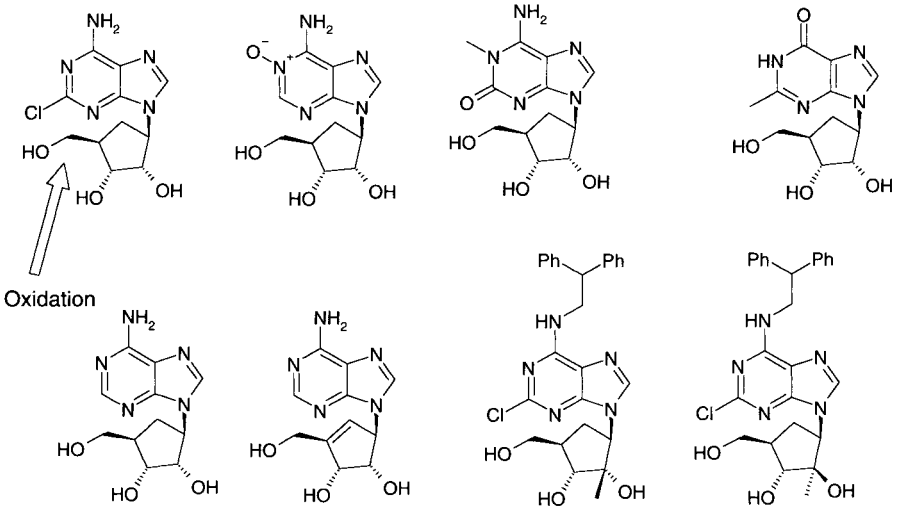


Figure 16.2-32. Non-natural substrates for nucleoside oxidase from *Pseudomonas* sp. These compounds are converted selectively to their corresponding 5'-carboxylic acids.

peroxide (yielding formate and carbon dioxide) and further metabolization by glycolate oxidase could be prevented by trapping the aldehyde function of glyoxylic acid as imine (Fig. 16.2-31)[136].

16.2.3.5
Nucleoside Oxidase (E.C. 1.1.3.28)

Nucleoside oxidase is produced by *Pseudomonas* species and related Gram negative bacteria[137]. The hetero-tetramer with covalently bound FAD oxidizes the 5'-hydroxyl group of purine and pyrimidine nucleosides to the corresponding carboxylic acids. It has found application in the analytical determination of nucleosides (e.g. in assessing food freshness)[138]. At Glaxo Wellcome R&D it found attention as key step in the production of anti-inflammatory compounds[139–141]. Several non-natural substrates were selectively converted on multi-gram scale into their 5'-carboxylic acids (Fig. 16.2-32).

The operational stability of the enzyme was improved by immobilization onto a solid matrix and especially by substitution of molecular oxygen as the primary electron acceptor by stoichiometric amounts of hydroquinone.

16.2.3.6
Glucose Oxidase (E.C. 1.1.3.4)

The most prominent of the alcohol oxidases is glucose oxidase. The dimeric flavoenzyme catalyzes the oxidation of β-D-glucose to D-glucono-δ-lactone, a reaction that has attracted the attention of generations of analytical chemists because of its

possible applicability in glucose sensors for diabetes control[142]. The reaction of the stoichiometrically formed hydrogen peroxide with various dyes can be used as the analytical signal[143]. More elegant variants (that at the same time avoid the formation of hazardous hydrogen peroxide) utilize anaerobic, electrochemical regeneration with a suitable mediator. Thus, the catalytic current becomes the analytical signal. Several approaches have been reported, e.g. the utilization of freely diffusible quinones[107], the incorporation of glucose oxidase in a conducting polymer (produced from 1,4-hydroquinones and soybean peroxidase), or the immobilization of several mediators in the vicinity of the prosthetic redox center[98, 99].

Because of the high substrate specificity of glucose oxidase, which almost exclusively accepts glucose (other substrates such as D-maltose, D-xylose, or L-sorbose are converted with less than 6% of the activity on glucose[144, 145]), this oxidase has not found any synthetic application, but it is frequently used in the food industry to remove traces of molecular oxygen from vacuum sealed products. Immobilized glucose oxidase is also used for the deoxygenation of juices and beer[146].

16.2.3.7
Alcohol Oxidase (E.C. 1.1.3.13)

The aliphatic alcohol oxidase, a FAD-dependent enzyme, catalyzes the oxidation of primary short-chain alcohols to the corresponding aldehydes. Dioxygen can be replaced by synthetic acceptors such as dichlorophenolindophenol or phenazine methosulfate[147].

By utilizing an alcohol oxidase from *Pichia pastoris* or *Candida* sp.[148], almost complete conversion of ethylene glycol into glyoxal (Fig. 16.2-30) was observed. These enzymatic routes were shown to be superior in terms of reaction conditions and yields compared to the chemical variants that make use of metal catalysts or even nitric acid for the oxidation of ethylene glycol.

Recently, aliphatic alcohol oxidase was applied as dehydrated enzyme in a gas-solid bioreactor[149]; an excess amount of catalase was added to prevent oxidase inactivation.

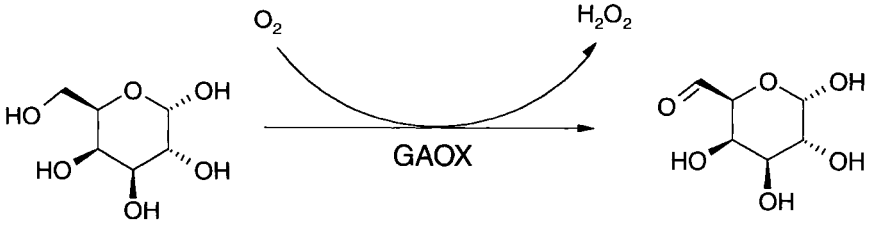


Figure 16.2-33. Galactose oxidase (GAOX) catalyzed oxidation of α-D-galactose to *meso*-galactohexodialose.

Table 16.2-9. Substrates and products of galactose oxidase.

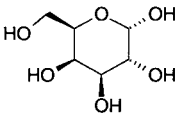
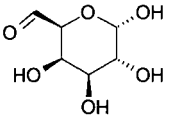
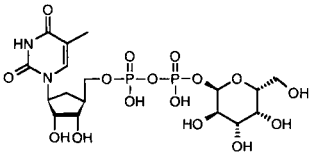
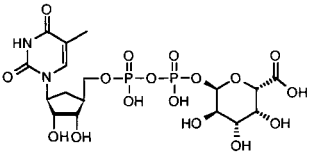
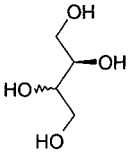
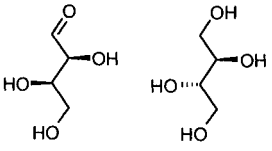
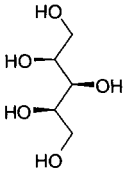
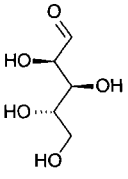
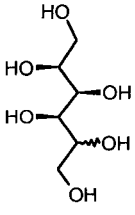
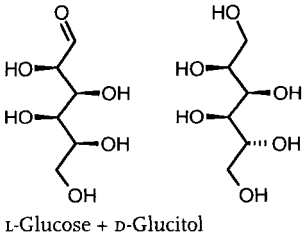
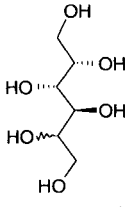
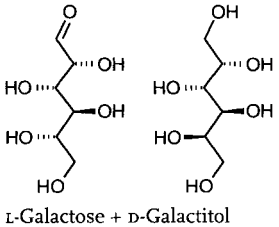
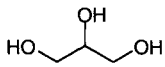
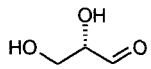
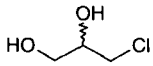
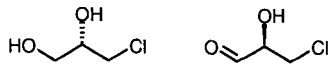
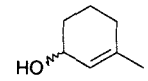
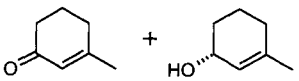
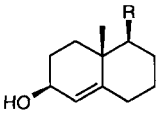
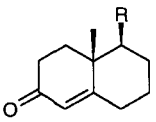
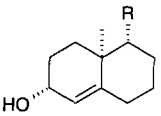
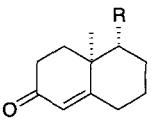
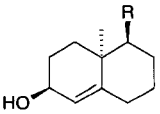
Substrate	Product	References
 D-Galactose	 *meso*-Galactohexodialdose	
 UDP-[^{14}C]-Galactose	 UDP-[^{14}C]-Galacturonic acid	[19]
 D,L-Threitol	 D-Threose + L-Threitol	[20]
 Xylitol	 L-Xylose	[20]
 D,L-Glucitol	 L-Glucose + D-Glucitol	[20]
 D,L-Galactitol	 L-Galactose + D-Galactitol	[20]

Table 16.2-9. (cont.).

Substrate	Product	References
	 L(-)Glyceraldehyde	[21]
	 (*S*)-Halodiol + (*R*)-Aldehyde	[21]

19 S. S. Basu, G. D. Dotson, C. R. H. Raetz, *Anal. Biochem.* **2000**, *280*, 173–177.
20 D. G. Drueckhammer, W. J. Hennen, R. L. Pederson, D. F. Barbas, C. M. Gautheron, T. Krach, C. H. Wong, *Synthesis* **1991**, *7*, 499–525.
21 A. M. Klibanov, B. N. Alberti, M. A. Marletta, *Biochem. Biophys. Res. Commun.* **1982**, *1982*, 108.

Table 16.2-10. Substrates and products in the kinetic resolution of allylic alcohols with cholesterol oxidase[22].

Substrate (R = H, OH)	Product
	
	
	
	No product detected

22 S. Dieth, D. Tritsch, J.-F. Biellmann, *Tetrahedron Lett.* **1995**, *36*, 2243–2246.

16.2.3.8
Galactose Oxidase (GAOX, E.C. 1.1.3.9)

Galactose oxidases belong to the group of copper-dependent oxidases. For the GAOX from *Dactylium dendroides* the existence of covalently bound pyrroloquinoline quinone (PQQ) could be shown[145]. It catalyzes the specific oxidation of the hydroxyl group in position 6 of galactose (Fig. 16.2-33)[150].

The enzyme regeneration can be performed aerobically or utilizing mediators

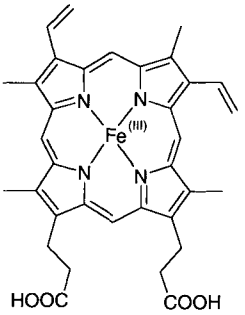


Figure 16.2-34. Ferric protoporphyrin IX as prosthetic group in most peroxidases.

such as ferrocene[102], tetracyano-iron-1,10-phenanthroline, or cobalt *tert*-pyridine complexes[103].

GAOX stereospecifically oxidizes a broad range of substrates (Table 16.2-9). In synthetic applications, the oxidation of racemic or *meso*-polyols such as D,L-threitol or xylitol to the non-native sugars are of special interest[151, 152]. In addition to the monosaccharides represented in Table 16.2-9, GAOX also converts di- or oligosaccharides[153].

16.2.3.9
Cholesterol Oxidase (ChOX, E.C. 1.1.3.6)

ChOX from *Rhodococcus erythropolis* was applied for the kinetic resolution of racemic mono- and bicyclic allyl alcohols (Table 16.2-10)[154]. Although the substrates tested were much smaller than the native substrate cholest-4-en-3β-ol, reasonable enantioselectivities (E) in the range of 7–20 were found for the (S) alcohols.

Both enantiomers of the alcohol (entry 1) were oxidized with moderate enantioselectivities (E = 7) for the (S) enantiomer. For bicyclic alcohols, the position of the hydroxyl group with respect to the methyl group is essential. Only at a relative *trans* configuration of both substituents significant oxidation occurred.

By utilizing organic redox dyes as primary electron acceptors and concomitant reoxidation at a glassy carbon electrode, amperometric biosensors for cholesterol based on cholesterol oxidase were developed[108].

16.2.4
Peroxidases as Catalysts

16.2.4.1
Introduction

Peroxidases (E.C. 1.11.1.7) are ubiquitously found in plants, microorganisms, and animals. Most peroxidases studied so far contain ferric protoporphyrin IX (protoheme, Fig. 16.2-34) as the prosthetic group[155]. However, some peroxidases also contain selenium (glutathione peroxidase)[156], vanadium (bromoperoxidase)[157],

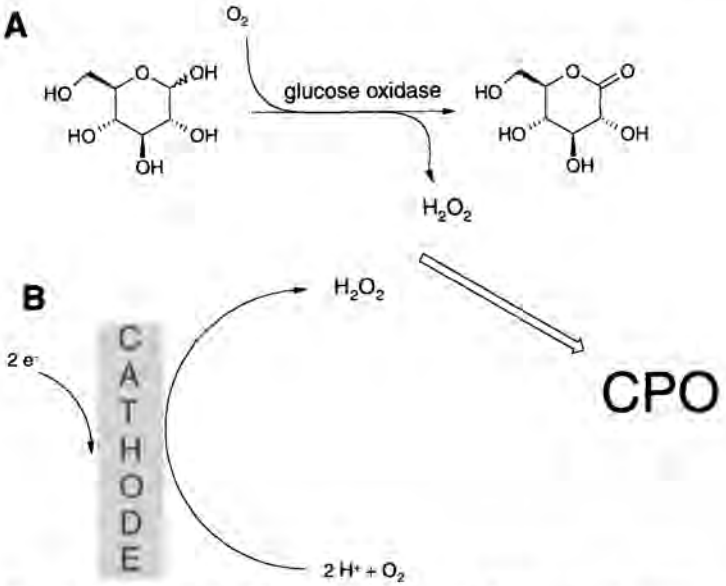


Figure 16.2-35. Methods of generating appropriate hydrogen peroxide concentrations for chloroperoxidase reactions, (A) enzymatically with glucose oxidase and (B) electrochemically by cathodic reduction of molecular oxygen.

manganese (manganese peroxidase)[158], and flavin (flavoperoxidase)[159] as prosthetic groups.

Most peroxidases accept a variety of peroxides, such as hydrogen peroxide or alkyl hydroperoxides, as oxidizing agents. The mechanism includes the activation of oxygen in a high valence iron-oxo species[155, 160].

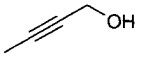
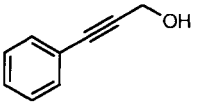
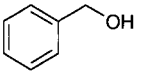
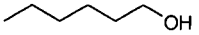
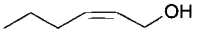
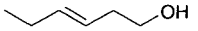
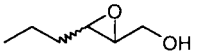
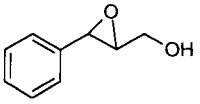
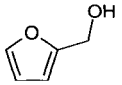
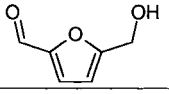
16.2.4.2
Methods to Generate H_2O_2

At a first glance, utilization of cheap hydrogen peroxide as electron acceptor seems appealing. The major drawback, however, is the sometimes rapid inactivation of peroxidases by their substrate. For example, chloroperoxidase (CPO, E.C. 1.11.1.10) exhibits a half-life time of 38 min even at an H_2O_2 concentration of 50 μM[161].

Several approaches to controlling hydrogen peroxide at a constant low concentration have been reported. In aqueous/organic emulsions, the use of *tert*-butyl hydroperoxide is beneficial. On the one hand, the peroxide concentration is limited according to the partition coefficient, and on the other hand, *tert*-butanol was shown to exert a stabilizing effect on CPO[162].

The slow continuous addition of hydrogen peroxide results in better CPO performance[163], which can be even further improved by sensor-controlled addition of H_2O_2[162], increasing the CPO total turnover number for indole oxidation more than 20-fold to ca. 860 000.

Table 16.2-11. Chloroperoxidase-catalyzed oxidation of some alcohols to the corresponding aldehydes.

Substrate	Yield [%]	Remarks and reference
	94	H_2O_2 or *tert*-butyl hydroperoxide as oxidants [23]
	95	H_2O_2 or *tert*-butyl hydroperoxide as oxidants [23]
	92	H_2O_2 or *tert*-butyl hydroperoxide as oxidants [23]
	quantitative	3 times higher activity with *tert*-butyl hydroperoxide in biphasic systems compared to H_2O_2 in buffer [24]
	81	[25]
	95	Production in gram-scale; low, non-enzymatic *cis/trans* isomerization observed [25]
	99	[25]
	97	[25]
	50 (40% *ee*)	Production in gram-scale, low yield with *cis*-isomer [25]
	46 (45% *ee*)	[25]
	92	[26]
	74	Quantitative conversion; significant amounts of acid as the product of overoxidation were found [26]

23 S. Hu, L. P. Hager, *Biochem. Biophys. Res. Commun.* **1998**, *253*, 544–546.
24 B. K. Samra, M. Andersson, P. Adlercreutz, *Biocat. Biotransf.* **1999**, *17*, 381–391.
25 E. Kiljunen, L. T. Kanerva, *J. Mol. Cat. B: Enzymatic* **2000**, *9*, 163–172.
26 M. P. J. van Deurzen, F. van Rantwijk, R. A. Sheldon, *J. Carbohydr. Chem.* **1997**, *16*, 299–309.

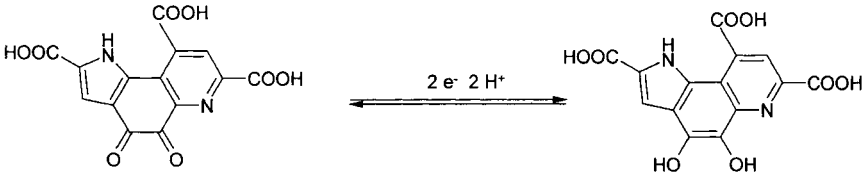


Figure 16.2-36. Pyrroloquinoline quinone (PQQ) in its oxidized and reduced form as prosthetic group for most quinoprotein dehydrogenases.

However, external H_2O_2 addition still has the disadvantage that locally high concentrations occur at the entry points, resulting in CPO inactivation at these hot spots. This can be circumvented via *in situ* generation of hydrogen peroxide. Two promising approaches have been reported so far: (i) another enzymatic reaction producing H_2O_2 e.g. with glucose oxidase[164], and (ii) electrochemical reduction of molecular oxygen (Fig. 16.2-35)[161, 165]. In both approaches, drastic increases of the number of CPO catalytic cycles up to 1.1×10^6 were achieved.

16.2.4.3
Chloroperoxidase (CPO, E.C. 1.11.1.10)

Publications on CPO-catalyzed oxidations of alcohols are rare. However, some selective oxidations of aliphatic, allylic, propagylic and benzylic alcohols to the aldehyde stage have been reported (Table 16.2-11).

16.2.4.4
Catalase (E.C. 1.11.1.6)

Most commonly, catalase is applied for the dismutation of hydrogen peroxide[166]. On reaction of catalase with one molecule of hydrogen peroxide, the intermediate high valence iron-oxo species is generated. This species, however, is a potent oxidant and readily reacts not only with a second molecule of hydrogen peroxide (yielding water and molecular oxygen) but has been reported to oxidize various other compounds such as methanol or nitrite[166].

Klibanov and coworkers enlarged the substrate spectrum by including a variety of alcohols that were oxidized to the corresponding aldehydes. Depending on the substrate and the reaction medium, high enantioselectivities are reported[167].

The generation of reactive catalase in its oxidized stage can also be achieved by direct electrochemical oxidation (transfer of electrons from ferric protoporphyrin IX to the electrode). Thus, catalase immobilized on graphite electrodes has been used for the hydrogen peroxide-free oxidation of phenol[168].

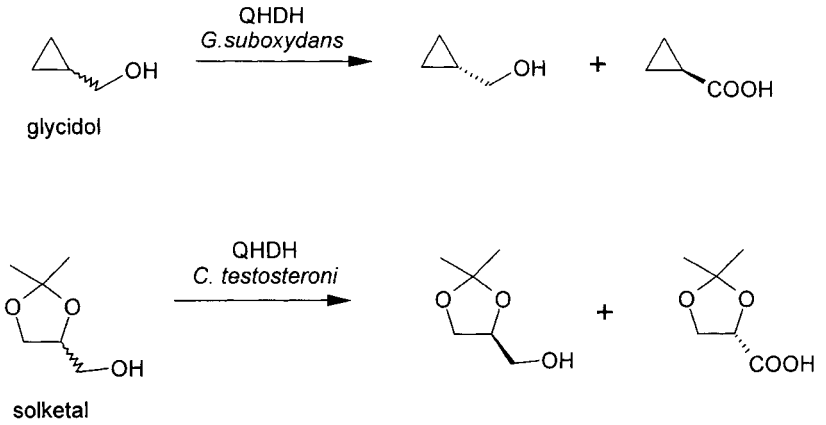


Figure 16.2-37. Resolution of alcohols by enantioselective oxidation using quinohemoprotein dehydrogenases (QHDH) from different microorganisms.

16.2.5
Quinoprotein Dehydrogenases (QDH)

16.2.5.1
General Remarks

Quinoproteins constitute a class of dehydrogenases distinct from the nicotinamide- and flavin-dependent oxidoreductases[169]. They use different quinone cofactors to convert a vast variety of alcohols and amines into their corresponding carbonyl products[170]. Proteins containing the cofactor pyrroloquinoline quinone (PQQ) (Fig. 16.2-36) form the largest and best-characterized sub-group.

QDHs are independent from classical coenzymes like $NAD(P)^+$. The substrate electrons are preferentially transferred to organic acceptors (quinones) and non-native redox mediators such as phenazine derivatives, DCPIP, Wursters blue[171], ferrocene[101], ferricyanide[172, 173], osmium complexes[174], or direct contact to an electrode[175].

One advantage of the PQQ-dependent dehydrogenases over the NAD(P)-dependent dehydrogenases is the more positive redox potential of the $PQQ/PQQH_2$ couple (+ 90 mV/pH 7[176] compared to – 320 mV[177, 178]).

Similarly to the flavin-dependent reactions, several mechanisms have been discussed, including covalent substrate-PQQ intermediates or hydride transfer[179–181]. The most important QDHs are methanol (alcohol) dehydrogenase (E.C. 1.1.99.8) and glucose dehydrogenase (E.C. 1.1.99.17), which will be discussed briefly.

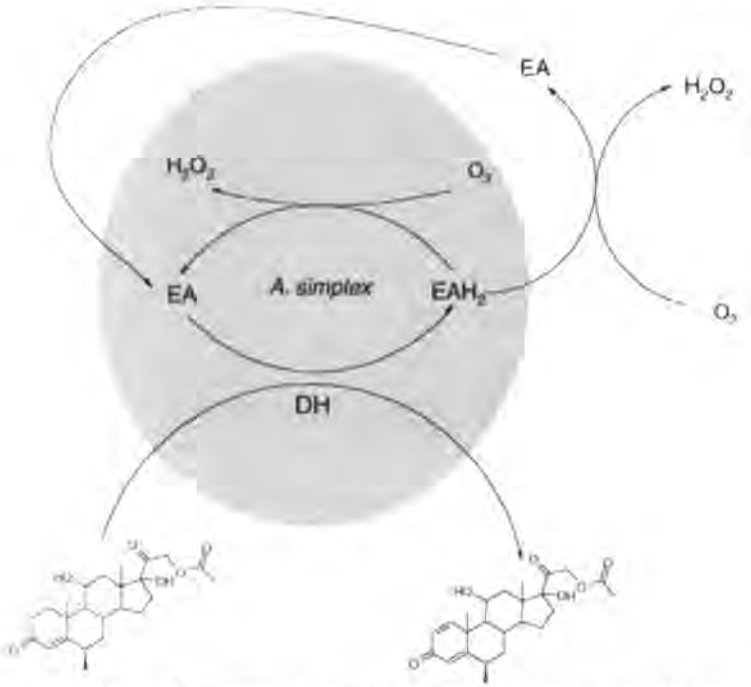


Figure 16.2-38. Δ^1-dehydrogenation of 6-α-methyl-hydrocortisone-21-acetate with polyurethane-entrapped *Arthrobacter simplex* cells in buffer-saturated 1-decanol. The dehydrogenase (DH) activity is largely increased on addition of quinoid electron acceptors (EA).

16.2.5.2

Methanol Dehydrogenase (E.C. 1.1.99.8)

In addition to PQQ, the methanol dehydrogenases from *Comamonas testosteroni* and *Gluconobacter suboxydans* contain a heme group, which is indicated in their synonym quinohemoprotein dehydrogenase.

The regeneration of these enzymes has been achieved by anodic reoxidation of ferricyanide[173], Os-modified anodes[174], or even direct contact to the anode[175].

Quinohemoprotein dehydrogenases (from *Comamonas testosteroni* and *Gluconobacter suboxydans*) have been reported to oxidize the alcohols solketal and glycidol (Fig. 16.2-37) enantioselectively[172].

Alcohol oxidases from various strains, and especially NAD(P) dependent dehydrogenases (except HLADH together with thio-NAD^+[182]), were found to be extremely inefficient for the oxidations in Figure 16.2-37, a fact, which is attributed to the significantly lower redox potential of the $NAD(P)^+/NAD(P)H$ redox system[172].

The QDH from *C. testosteroni* was further characterized[183]. It oxidizes stereospecifically the (*R*) enantiomer of secondary alcohols. Both, k_{cat}/K_M and *E* increased with the substrate chain length. *In vitro*, ferricyanide was used as sacrificial electron acceptor. *In vivo*, the excess electrons are most probably transferred to molecular

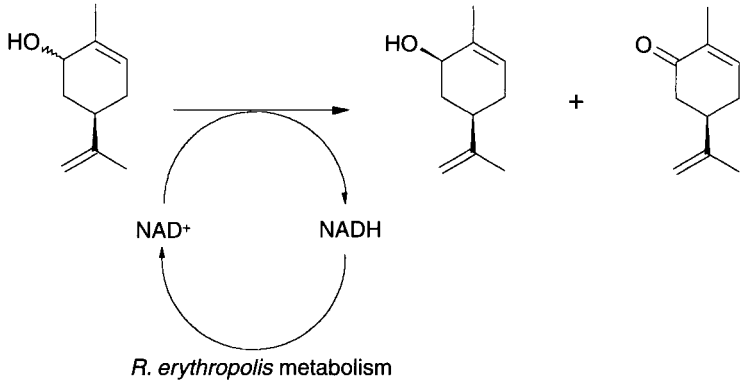


Figure 16.2-39. Enantiospecific oxidation of racemic carveol to (–)-carvone and (–)-*cis* carveol using whole cells of *Rhodococcus erythropolis*.

oxygen via the respirator chain. This process is considerably accelerated (by a factor of 12) upon addition of external quinoid electron acceptors such as vitamin K (that are capable of autoregeneration) (Fig. 16.2-38)[184].

16.2.5.3
Glucose Dehydrogenase (E.C. 1.1.99.17)

So far, a membrane-bound[185] and a soluble glucose dehydrogenase[186] have been identified. The latter oxidizes a wide range of mono- and disaccharides[186]. In addition to cytochrome b_{562}, regeneration with artificial acceptors such as DCPIP or ferrocene[187, 188] is effective and unproblematic, as no autoregeneration with molecular oxygen (producing reactive O-species) is possible. It has commercial interest as a component of glucose test strips for diabetes control[189].

16.2.6
Whole-Cell Oxidations

16.2.6.1
Stereoselective Oxidation of (–)-Carveol to (–)-Carvone[190]

By using whole cells of *Rhodococcus erythropolis* DCL14, a racemic mixture of (–)-carveol was converted to (–)-carvone and (–)-*cis*-carveol (Fig. 16.2-39). The system was optimized using the two-liquid concept, in which a second organic phase serves as substrate and product reservoir. (–)-Carvone is an important flavor compound.

The enzyme responsible for this bioconversion, catalyzed by wild-type cells of *Rhodococcus erythropolis* DCL14, is carveol dehydrogenase[191]. A high enantioselectivity and no further conversion of (–)-carvone was obtained. Carveol dehydrogenase has a broad substrate specificity and prefers substituted cyclohexanols as substrates[191]. The regeneration of the cofactor NAD^+ was accomplished by the use of living cells.

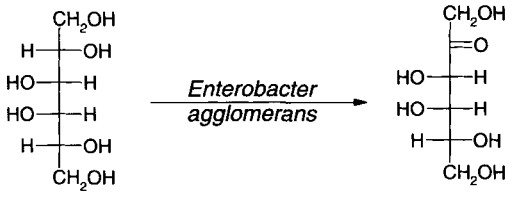


Figure 16.2-40. Production of the low calorie sweetener tagatose from D-galactitol by whole cells of *Enterobacter agglomerans*.

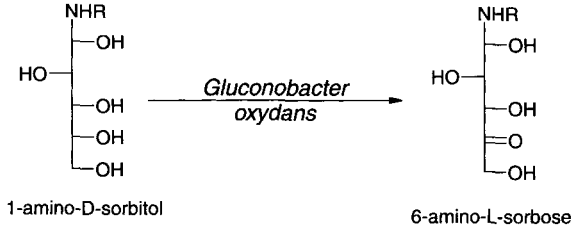


Figure 16.2-41. Oxidation of N-protected 1-amino-D-sorbitol to 6-amino-L-sorbose using *Gluconobacter oxydans*.

The use of a two-liquid phase system consisting of a 1:1 mixture of phosphate buffer and dodecane resulted in an increase of the initial (–)-*trans*-carveol conversion rate by 70% (to 26 nmol per minute and per mg protein). The production was increased from 4.3 to 208 μmol (–)-carvone formed per mg protein as compared to the aqueous system. A simple downstream process consisting of phase separation, methanol extraction, evaporation, and separation of (–)-*cis*-carveol and (–)-carvone over a silica gel column, was developed.

In another study, *Rhodococcus globerulus* PWD8 was found to oxidize D-limonene regio- and enantioselectively via (+)-*trans*-carveol to (+)-carvone[192].

16.2.6.2
Sugar Dehydrogenases Applied in Whole Cells

Cofactor regeneration by the cell metabolism is the main advantage of whole cells in polyalcohol oxidations. The induction of whole-cell biocatalyst activity is dependent on the nature of the growth substrate. An example is the production of the low calorie carbohydrate sweetener tagatose from D-galactitol (Fig. 16.2-40). As biocatalysts, wild-type strains of *Enterobacter agglomerans* and *Gluconobacter oxydans* DSM 2343, in which sugar dehydrogenases catalyze the reaction of interest, were described[193, 194].

In the case of *Enterobacter agglomerans*, cells growing on 1% glycerol plus 1% erythritol resulted in the best biocatalytic performance. In 30 h, galactitol (50 g/L) was converted with a tagatose yield of 86%. Immobilization and storage at – 20 °C are possible.

With *Gluconobacter oxydans*, growing cells were found to be more effective than resting cells. Furthermore, galactitol adaptation gave a notable increase in tagatose yield.

Another example is the oxidation of 1-amino-D-sorbitol (N-protected) to 6-amino-L-sorbose (Fig. 16.2-41)[195]. This reaction was published as a step in the synthesis of

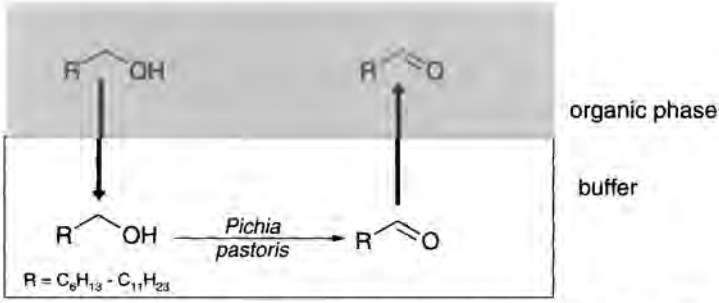


Figure 16.2-42. Selective oxidation of linear and branched aliphatic alcohols to the corresponding aldehydes using *P. pastoris* in aqueous/organic reaction mixtures.

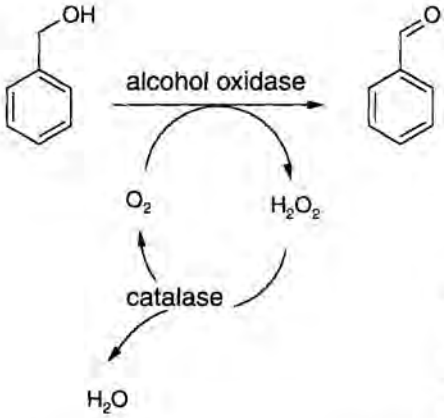


Figure 16.2-43. Oxidation of benzylic alcohol to benzaldehyde using whole cells of *P. pastoris* in organic/aqueous emulsions or with purified alcohol oxidase. *In vitro* hydrogen peroxide was removed by catalase.

1-desoxynojirimycin. Derivatives of 1-desoxynojirimycin are pharmaceuticals for the treatment of carbohydrate metabolism disorders (e. g. diabetes mellitus). Suspended whole cells of *Gluconobacter oxydans* were used as the biocatalyst, in which D-sorbitol dehydrogenase is responsible for this biotransformation.

To prevent undesired follow-up reactions of 6-amino-L-sorbose in water, the amino group has to be protected by, for example, a benzyloxycarbonyl group (*R*). Cells are produced by fermentation on sorbitol and used for the bioconversion step as resting cells in water without added nutrients. The biotransformation is carried out by Bayer in a 10 000 L reactor with 90 % yield.

16.2.6.3
Oxidation of Aromatic and Aliphatic Alcohols to Corresponding Aldehydes and Acids

Flavin-containing alcohol oxidase combined with catalase in peroxisomes of *Pichia pastoris* naturally catalyzes the oxidation of methanol to formaldehyde. *In vivo* and *in vitro* applications are possible. The alcohol oxidase has a broader substrate specificity than the subsequent enzymes of the methanol degradation pathway. Therefore,

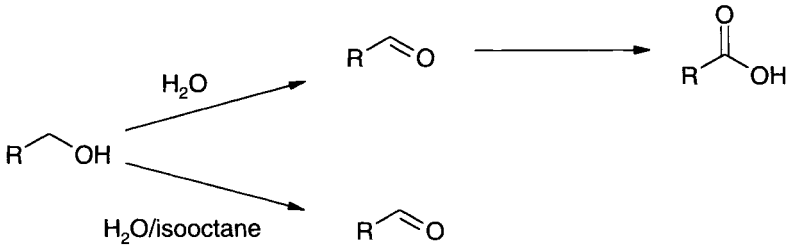


Figure 16.2-44. Oxidation of alcohols by whole cells of *Acinetobacter*. In aqueous media the oxidation proceeds until the acid stage, whereas the aldehyde is accumulated in the presence of organic solvents.

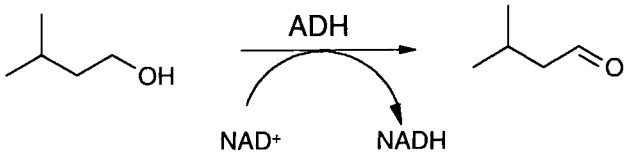


Figure 16.2-45. Preparation of isovaleraldehyde using an alcohol dehydrogenase (ADH) in whole cells of *Gluconobacter oxydans*.

products other than formaldehyde are not degraded further. The spectrum of alcohols oxidized by whole cells of *Pichia pastoris* includes aliphatic C_1-C_5 alcohols (saturated, unsaturated or branched). In biphasic media, *Pichia pastoris* also oxidizes C_6-C_{11} alcohols, phenylethyl alcohol and 3-phenyl-1-propanol[196].

Up to 70 g/L acetaldehyde was produced from ethanol[197–199]. Here, competitive product inhibition was partially overcome by high Tris buffer concentrations. Tris is able to bind acetaldehyde and markedly improve reaction yields. In a biphasic system consisting of 97 % hexane and 3 % aqueous phase, hexanol (11 g/L) was converted to hexanal (Fig. 16.2-42) within 24 h at a yield of 96 %[196]. In another example, benzyl alcohol was oxidized by whole cells of *Pichia pastoris* and purified alcohol oxidase (Fig. 16.2-43)[200]. For this reaction the importance of solute partitioning in the biphasic reaction system was studied[201]. With immobilized cells in organic (xylene)/aqueous media, benzaldehyde concentrations up to 30 g/L were reached in the organic phase[201]. With purified alcohol oxidase, up to 45 g/L benzaldehyde was produced within 8 h and with an enzyme concentration of 0.94 g/L.

Dehydrogenases of Acinetobacter and Gluconobacter strains catalyze the oxidation of various alcohols to corresponding aldehydes and acids *in vivo*. Substrates tested

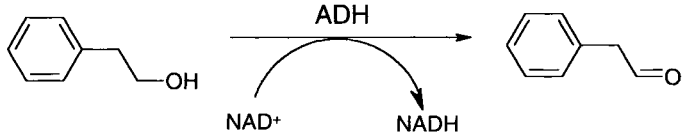
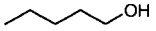
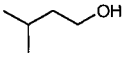
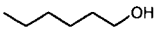
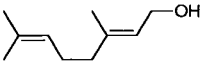
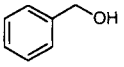
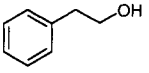
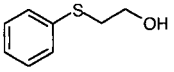
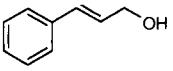
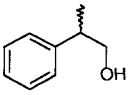


Figure 16.2-46. Preparation of 2-phenyl acetaldehyde with *Acinetobacter* sp. in organic/aqueous emulsions.

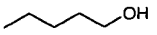
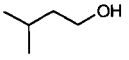
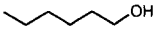
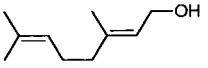
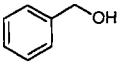
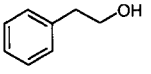
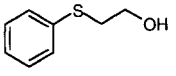
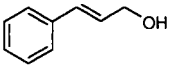
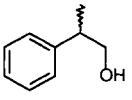
Table 16.2-12. Oxidations catalyzed by *Acinetobacter* sp. in aqueous and biphasic media[27].

	Water		Water/isooctane (vol/vol 1/1)	
Substrate	**Acid yield [%]**	**Time [h]**	**Aldehyde yield [%]**	**Time [h]**
	> 97	3	74	1
	> 97	3	90	1
	> 97	3	87	1
	> 97	24	72	4
	25	24	< 5	24
	> 97	3	90	45 min
	> 97	2	93	45 min
	> 97	8	77	45 min
 racemic	40 ((*S*)-alcohol: 95% *ee*)	24	< 5	24

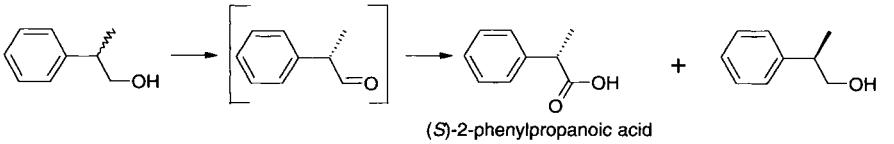
27 R. Gandolfi, N. Ferrara, F. Molinari, *Tetrahedron Lett.* **2001**, *42*, 513–514.

include ethanol, propanol, butanol, 2-methyl-1-butanol, 3-methyl-1-butanol, 1-pentanol, 1-hexanol, geraniol, 2-phenylethanol, 2-phenylthioethanol, cinnamyl alcohol, benzyl alcohol and (*R*,*S*)-2-phenyl-1-propanol (Tables 16.2-12 and 16.2-13)[202, 203]. The molecular structure of substrates and products as well as physicochemical conditions significantly influence bioconversions of short-chain aliphatic and aromatic alcohols into acids[204, 205]. Yields depend on the toxicity of the alcohol (different inhibitory concentrations for different alcohols), since product inhibition is often the major limiting factor[204]. Furthermore, dissolved oxygen concentrations and pH conditions are important factors for improving such bioconversions. Depending on strain and substrate (specificity of dehydrogenases), the reaction is directed to aldehyde *or* acid accumulation (Fig. 16.2-44). In principle, acid accumulation is favored in aqueous media, whereas aldehydes preferentially accumulate in biphasic media[203].

Table 16.2-13. Oxidations catalyzed by *Gluconobacter asaii* in aqueous and biphasic systems[27].

Substrate	Water		Water/isooctane (vol/vol 1/1)	
	Acid yield [%]	Time [h]	Aldehyde yield [%]	Time [h]
	> 97	4	93	45 min
	> 97	4	90	1
	> 97	3	91	45 min
	16	24	29	5
	< 5	24	< 5	24
	> 97	5	85	2
	> 97	5	96	1
	20	24	24	4
 racemic	33	24	< 5	24

27 R. Gandolfi, N. Ferrara, F. Molinari, *Tetrahedron Lett.* **2001**, *42*, 513–514.



(*S*)-2-phenylpropanoic acid

Figure 16.2-47. Resolution of racemic (*R,S*)-2-phenylpropionic alcohol with whole cells of *Gluconobacter oxydans* yielding (*S*)-2-phenylpropanoic acid and (*R*)–2-phenylpropionic alcohol.

An example of aldehyde formation is the production of isovaleraldehyde by *Gluconobacter oxydans* R (Fig. 16.2-45)[202, 206]. Glycerol-grown *Gluconobacter oxydans* slowly oxidizes 3-methyl-1-butanol to isovaleraldehyde, with yields of over 90%. The product was recovered by bisulphite trapping or cold traps[202]. Extractive bioconversion in a hollow-fiber membrane bioreactor allowed continuous produc-

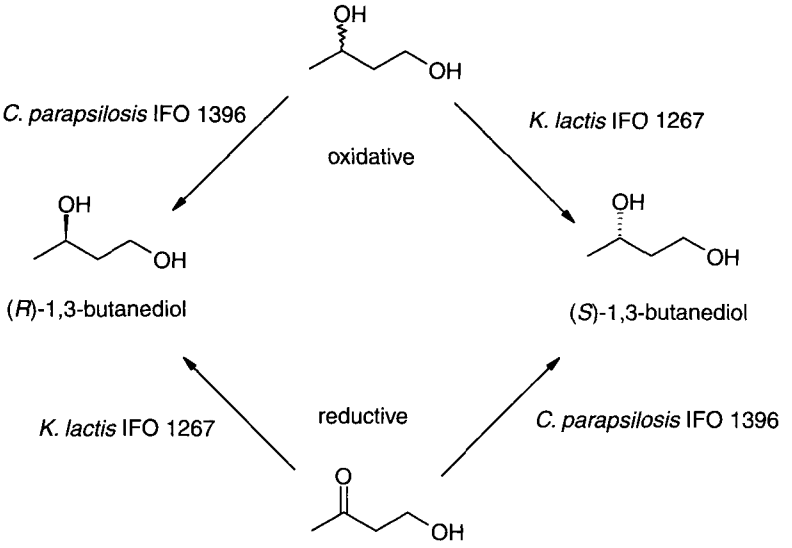


Figure 16.2-48. Preparation of both enantiomers of 1,3-butanediol with whole cells of *K. lactis* and *C. parapsilosis* either by enantioselective oxidation of 1,3-butanediol (oxidative) or enantioselective reduction of 4-hydroxybutanone (reductive).

tion of isovaleraldehyde at overall productivities of 2–3 g L^{-1} h^{-1} [206]. Yields between 72 and 90% were reached.

Another example of a synthesis is the production of phenylacetaldehyde using *Acinetobacter* strains (Fig. 16.2-46) [207, 208]. Different two-liquid phase systems were tested for their ability to remove the aldehyde into the organic phase before its further conversion to acid. In an optimized two-liquid-phase process, in which isooctane (at a volume fraction of 50%) was used as the organic carrier solvent, product concentrations of 9 g/L were reached in 4 h of reaction, corresponding to a yield of 90% [208]. The production strain *Acinetobacter* sp. ALEG showed satisfactory long-term stability, being able to perform the transformation with 80% of the original activity after 3 days of contact with the solvent.

Besides the multigram-scale production of different aliphatic carboxylic acids by biocatalytic alcohol oxidation, especially the enantioselective oxidation of racemic 2-phenyl-1-propanol to (*S*)-2-phenylpropanoic acid with *Gluconobacter oxydans* (Fig. 16.2-47) is another good example of acid production from alcohols [209].

After optimization of the parameters temperature, pH, substrate concentration, and agitation speed using a simplex sequential method, the resolution involving two oxidation steps yielded 45% product with an *ee* of 98%.

16.2.6.4
Enantiospecific Reactions

Two ways of producing *optically pure 1,3-butanediol via microbial resolution* have been reported: the oxidation of a racemic mixture of 1,3-butanediol yielding one enantio-

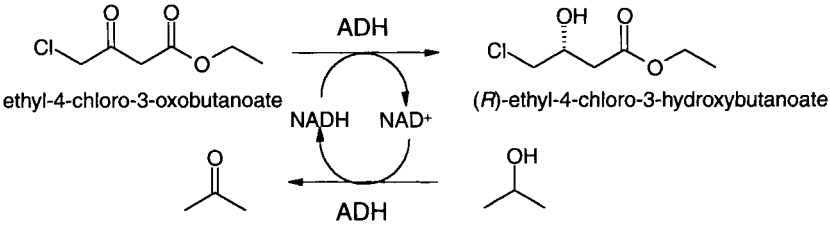


Figure 16.2-49. Asymmetric reduction of ethyl-4-chloro-3-oxobutanoate catalyzed by an alcohol dehydrogenase (ADH) in recombinant *E. coli*. The necessary reduction equivalents were derived from the oxidation of isopropanol with the same enzyme.

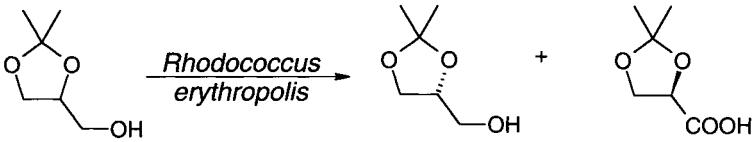


Figure 16.2-50. Enantioselective oxidation of isopropylideneglycerol utilizing *Rhodococcus erthyropolis*.

mer and 4-hydroxy-2-butanone, and the reduction of the 4-hydroxy-2-butanone yielding one enantiomer of 1,3-butanediol (Fig. 16.2-48). (*R*)-1,3-butanediol is an important chiral synthon for the synthesis of various optically active compounds such as azetidinone derivatives, which are intermediates in the production of antibiotics, pheromones, fragrances, and insecticides.

From a screening procedure, *Kluyveromyces lactis* IFO 1903 and *Candida parapsilosis* IFO 1396 were found to be effective in the enantioselective oxidation of (*R*)-1,3-butanediol and (*S*)-1,3-butanediol, respectively, and in the asymmetric reduction of 4-hydroxy-2-butanone to (*R*)-1,3-butanediol and (*S*)-1,3-butanediol, respectively[210].

The equilibria between ketones and alcohols are catalyzed by secondary alcohol dehydrogenases. The secondary alcohol dehydrogenase of *C. parapsilosis* IFO 1396

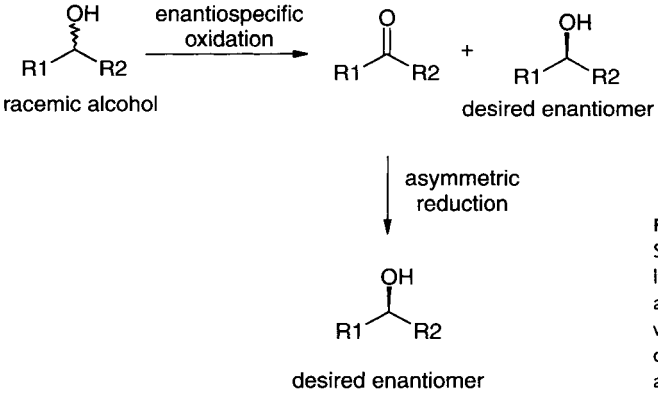
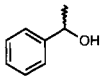
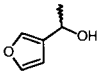
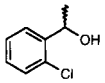
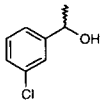
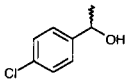
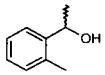
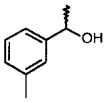
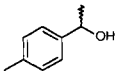
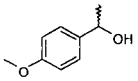


Figure 16.2-51. Stereoinversion catalyzed by two different alcohol dehydrogenases via enantiospecific oxidation followed by an asymmetric reduction.

was purified and characterized as an NAD^+-dependent dehydrogenase with a broad substrate specificity (secondary alcohols > primary alcohols)[211]. The alcohol dehydrogenase gene of *C. parapsilosis* was cloned and expressed in recombinant *E. coli* JM109, which showed more than twofold higher specific alcohol dehydrogenase activity than *C. parapsilosis*[212].

Resting cells of *C. parapsilosis* were used for the large-scale (2000 L) production of (*R*)-1,3-butanediol (94% *ee*) from racemic 1,3-butanediol. After down-stream processing 3.1 kg product was isolated (overall yield: 15.5%), and a chemical purity of

Table 16.2-14. Biocatalytic stereoinversions with *Geotrichum candidum*[28].

	Without allyl alcohol			With allyl alcohol (33 mM)		
Substrate	**Yield [%]**	***ee* [%]**	**Configuration**	**Yield [%]**	***ee* [%]**	**Configuration**
	96	99	(*R*)	94	98	(*R*)
	65	92	(*R*)	57	86	(*R*)
	99	3	(*S*)	100	0	–
	90	16	(*R*)	85	23	(*R*)
	97	89	(*R*)	97	96	(*R*)
	99	2	(*R*)	89	21	(*R*)
	89	21	(*R*)	55	94	(*R*)
	95	79	(*R*)	74	96	(*R*)
	77	97	(*R*)	54	99	(*R*)

28 K. Nakamura, Y. Inoue, T. Matsuda, A. Ohno, *Tetrahedron Lett.* **1995**, *36*.

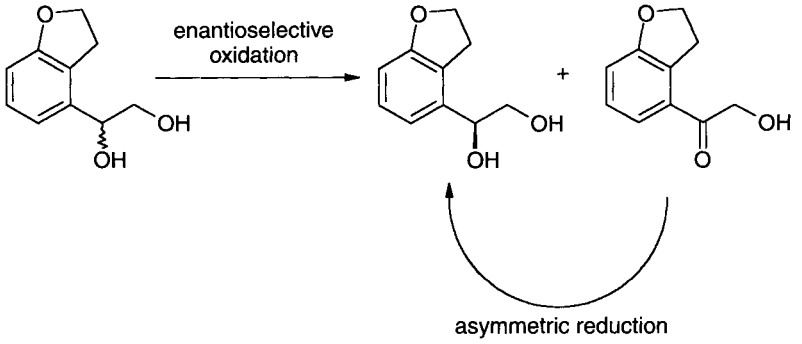


Figure 16.2-52. Synthetic application of the stereoinversion concept using *Candida* sp. and *Pichia* sp.

98.8% was reached[213]. Resting cells of recombinant *E. coli* were reported to produce (*R*)-1,3-butanediol (93.5% *ee*, 94.7% yield) from the racemate without any additive to regenerate NAD^+ from NADH[212].

In another application, recombinant *E. coli* produced 36.6 g/L ethyl-(*R*)-4-chloro-3-hydroxybutanoate (99% *ee*) from 40 g/L ethyl-4-chloro-3-oxo-butanoate[210]. Here, the secondary alcohol dehydrogenase served as both synthetic (asymmetric reduction) and regenerating (NADH-regeneration via isopropanol oxidation) enzyme (Fig. 16.2-49).

Enzymatic resolution of (R/S) isopropylideneglycerol[214, 215]

Whole cells of *Rhodococcus erythropolis* were used for the selective oxidation of the (*S*)-enantiomer of isopropylideneglycerol (Fig. 16.2-50). With a 50% conversion of the racemate, an *ee* value of over 98% was reached for (*R*)-isopropylideneglycerol and of over 90% for (*R*)-isopropylideneglyceric acid.

(*R*)-Isopropylideneglycerol is a useful C_3-synthon in the synthesis of (*S*)-β-blockers; e.g. (*S*)-metoprolol. (*R*)-Isopropylideneglyceric acid can also be used as starting material for the synthesis of biologically active compounds.

16.2.6.5
Stereoinversions using Microbial Redox Reactions[216]

Racemic mixtures of secondary alcohols can be resolved completely by enantiospecific enzyme-catalyzed oxidation resulting in one enantiomer of the alcohol and the ketone followed by asymmetric enzyme-catalyzed reduction of the ketone (Fig. 16.2-51). For oxidation and reduction, two separate microorganisms[217–219] or two different enzymes in a single microorganism[220–222] may be used.

An example of a suitable biocatalyst is *Geotrichum candidum*, harboring both an oxidizing and a reducing enzyme activity. Table 16.2-14 shows the catalytic performance of *Geotrichum candidum* towards different substrates[220] when the biocatalyst is incubated for 24 h with 27 mM substrate. Allyl alcohol effectively shifts the stereoselectivity of the reduction. It is presumed to inhibit enzyme(s) that reduce aryl

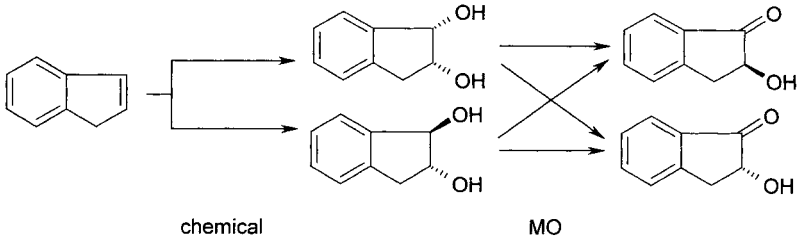


Figure 16.2-53. Chemoenzymatic synthesis of 2-hydroxy-1-indanone. The racemic *syn* and *anti* diols were prepared by chemical dihydroxylation of indane. Asymmetric induction was achieved by microbial oxidation (MO) of these diols.

Table 16.2-15. Substrate spedificity of *Arthrobacter* and *Pseudomonas* strains [29].

Taxonomy	Substrate specificity (no substrates)
Arthrobacter sp. strain 1HB	*cis*-(1*S*, 2*R*)-diol > *trans*-(1*S*, 2*S*)-diol >> (*cis*-(1*R*, 2*S*)-diol, *trans*-(1*R*, 2*R*)-diol)
Arthrobacter sp. strain 1HE	*cis*-(1*S*, 2*R*)-diol >> *trans*-(1*S*, 2*S*)-diol >> (*cis*-(1*R*, 2*S*)-diol, *trans*-(1*R*, 2*R*)-diol)
Pseudomonas aeruginosa strain IN	*trans*-(1*R*, 2*R*)-diol > *cis*-(1*S*, 2*R*)-diol > *cis*-(1*R*, 2*S*)-diol >> (*trans*-(1*S*, 2*S*)-diol)

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Table 16.2-16. Microbial stereoselective oxidation of *cis*- and *trans*-1,2-indandiols [29].

Strain	Substrate	Product	Reaction time [h]	Yield [%]	*ee* [%]
Arthrobacter sp. 1HB	*Cis*	R	4	46	> 99.9
	Trans	S	12	35	> 99.9
Arthrobacter sp. 1HB	*Cis*	R	4	47	> 99.9
	Trans	S	24	8	> 99.9
P. aeruginosa IN	*Cis*	R	5	7	82.5
	Trans	R	24	40	> 99.9

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methyl ketone to (*S*)-1-arylethanol. The inhibition of yeast reductases by allyl alcohols has been reported [223].

Another example is the deracemization of (*RS*)-1-{2',3'-dihydrobenzo[*b*]furan-4'-yl}-ethane-1,2-diol by biocatalytic stereoinversion (Fig. 16.2-52) [224]. In order to find an appropriate biocatalyst to accomplish such a deracemization, different microorganisms were screened. Several microorganisms belonging to the genera *Candida* and *Pichia* allowed yields of 60–70% with 90–100% enantiomeric excess. Substrate dissolved in DMF was added to the biotransformation mixture consisting of resting cells suspended in phosphate buffer (pH 7). The presence of glucose generally increased the yield but lowered the enantiomeric excess. Different microorganisms can be suitable for a given stereoinversion and the optimal biocatalyst should be chosen by screening.

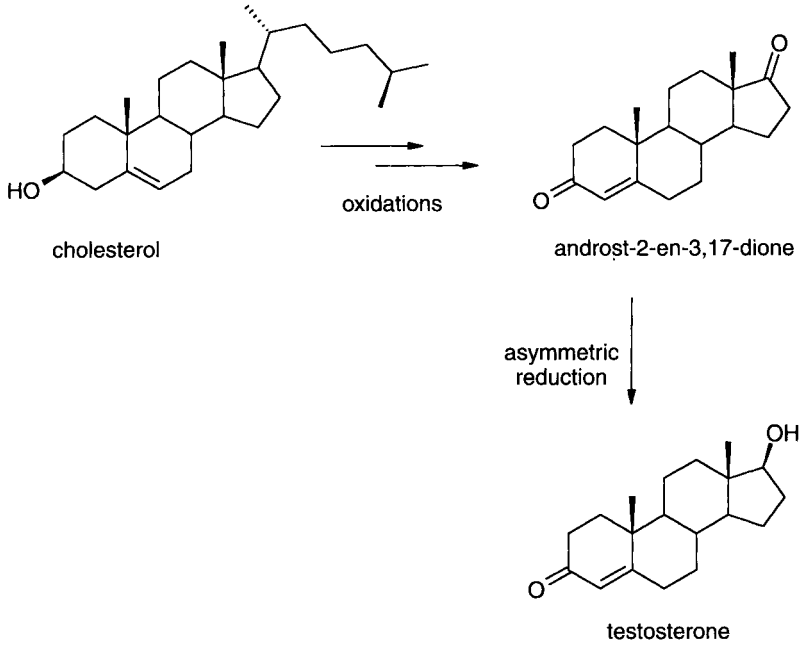


Figure 16.2-54. Selective oxidation of cholesterol to testosterone by whole cells of *Mycobacterium* sp NRRL B-3805.

Stereoselective oxidation of racemic 1,2-indandiols[225]

Kato *et al.* described the stereoselective microbial synthesis of both enantiomers of 2-hydroxy-1-indanone, selecting *cis*- or *trans*-diol as the substrate (Fig. 16.2-53). *Cis*-1-amino-2-indanol is an important synthon in organic chemistry (for example in the synthesis of the leading HIV protease inhibitor Crixivan) and can easily be synthesized from optically active 2-hydroxy-1-indanone[226].

Microorganisms degrading indane derivatives were screened for stereoselective oxidation of racemic *cis*- or *trans*-1,2-indandiol. Three promising strains specifically oxidizing the benzylic hydroxyl group were found (see Table 16.2-15).

All strains produced inducible enzymes responsible for the oxidation reaction, recognizing the stereochemistry of the 1- or 2-positions of the diol regardless of their *cis* and *trans* geometry. By using the resting cells of the strains, both enantiomers of 2-hydroxy-1-indanone were synthesized in enantiomerically pure form simply by selecting *cis*- or *trans*-1,2-indandiol as the substrate. Growth conditions were optimized to promote cell growth and the formation of 1,2-indanediol-oxidizing activity. The biocatalyst activity was optimally induced with 0.05% indanol. Carefully choosing appropriate carbon and nitrogen sources is crucial for optimal biocatalyst activity and cell growth.

Table 16.2-16 shows the stereoselective oxidation of racemic *cis*-diol or *trans*-diol into optically active 2-hydroxy-1-indanone at a 2 mL scale with 50 mg dry cells per ml.

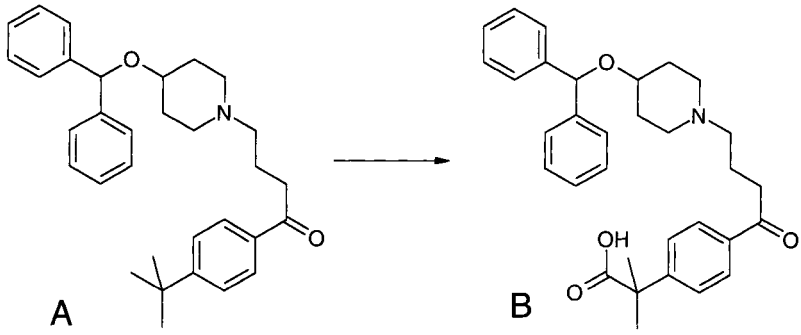


Figure 16.2-55. Regioselective three-step oxidation of ebastine (A) to carebastine (B) using *Cunninghamella blakesleeana*.

Production of testosterone from cholesterol using Mycobacterium sp.[227]
In this multistep reaction the microbial degradation of sterol side chains combined with the reduction of an intermediate thereof is used to accumulate testosterone from cholesterol. A cholesterol-assimilating and androst-2-en-3,17-dione-accumulating mutant of *Mycobacterium* sp. NRRL B-3805 oxidizes cholesterol through multiple steps of the sterol side chain degradation pathway, also involving alcohol oxidations, to androst-2-en-3,17-dione (Fig. 16.2-54). This multistep oxidation is followed by the reduction of androst-2-en-3,17-dione to testosterone by the NADH requiring activity of 17β-hydroxysteroid dehydrogenase. This activity is dependent on the presence of glucose as the carbon source. After the glucose in the fermentation culture is completely consumed, most testosterone is oxidized to androst-2-en-3,17-dione. Adding a larger amount of glucose prevents this oxidation.

On a 2.5 L scale a yield of 51 % was reached in 120 h of cultivation. Here, the initial substrate concentration amounted to 0.1 % (w/v).

Microbial oxidation of ebastine[228]
Ebastine is a new generation antihistaminic drug with fewer side-effects. The microbial three-step oxidation of ebastine, using whole cells of the mold *Cunninghamella blakesleeana* as biocatalysts, involves an alcohol and an aldehyde oxidation step and results in the formation of carebastine, which is the pharmacologically active compound[229]. The initial step in the oxidation of ebastine is hydroxylation by a cytochrome P-450-dependent monooxygenase to the corresponding alcohol. The two consecutive oxidations are catalyzed by oxidoreductases, which are not further characterized, and lead via the aldehyde to the corresponding carboxylic acid carebastine (Figure 16.2-55).

Growth in a complex medium containing soybean-peptone and yeast extract is necessary for biocatalyst activity. A component of soybean-peptone, genistein, is thought to act as an inducer of cytochrome P-450 enzymes. Growing cells provide a higher yield than resting cells. Addition of 1% poly(vinyl alcohol) was found to prevent pellet formation and thereby to guarantee constant mass transfer rates.

From a 3 L batch fermentation, 270 mg carebastine was isolated (yield: 45 %).

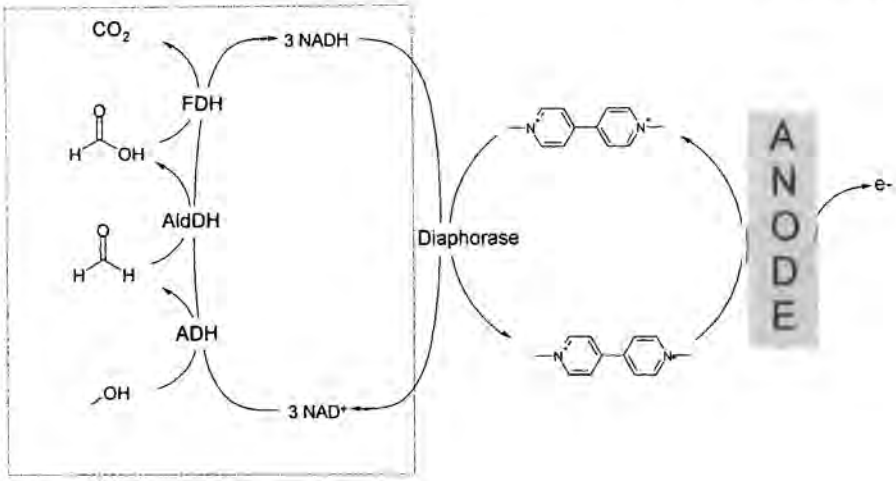


Figure 16.2-56. Enzymatic three-step oxidation of methanol to carbon dioxide in the anodic compartment of a biofuel cell.

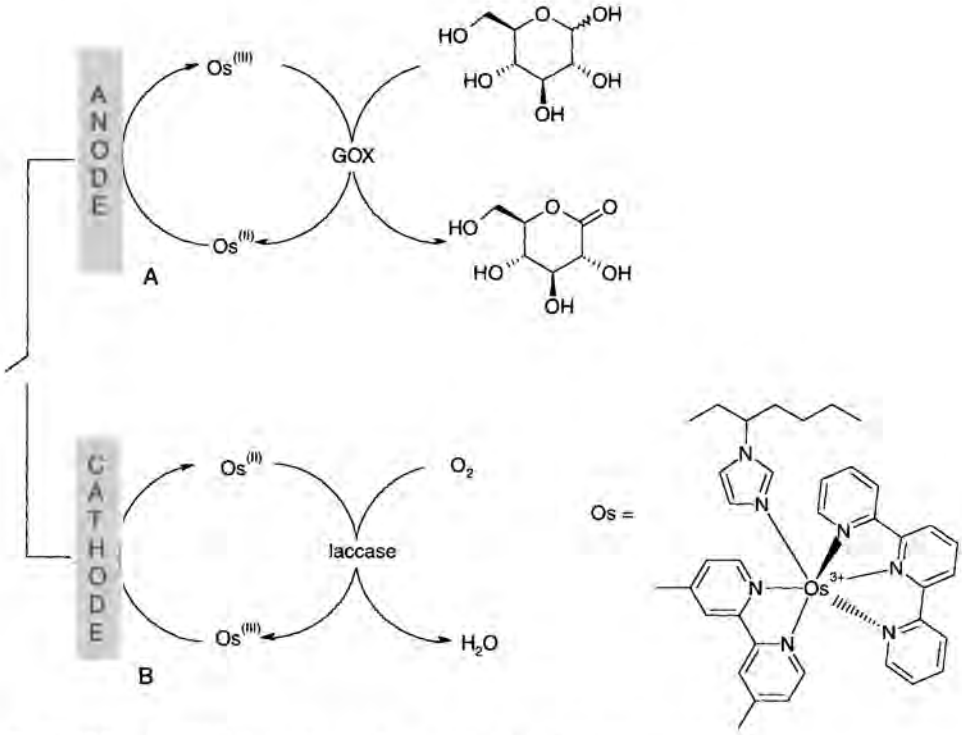


Figure 16.2-57. Mediated electron transfer steps in the electroenzymatic oxidation of glucose (A) and reduction of O_2.

Therefore, after 24 h of cultivation, 600 mg ebastine was added and the incubation was continued for 68 h.

16.2.7 Miscellaneous

16.2.7.1 Biofuel Cells

In recent years, biofuel cells have gained tremendous attention. The use of methanol instead of dihydrogen as the oxidizable substance offers special advantages as it is readily available and easy to store and handle. At the same time, the theoretical cell voltage of an $MeOH/O_2$ cell (1.19 V) is near that of H_2/O_2 (1.23 V).

Whitesides and coworkers recently developed a biofuel cell based on the step-wise enzymatic oxidation of methanol to carbon dioxide (Fig. 16.2-56)[230]. In the anodic compartment of the biofuel cell, methanol is oxidized to carbon dioxide in three steps: by an alcohol dehydrogenase, an aldehyde dehydrogenase, and ultimately formate dehydrogenase. In each of these enzymatic steps, one equivalent of NADH is produced. NADH itself transfers its electrons via diaphorase to viologene and in the end to the anode. The redox potential of the reduced/oxidized viologene couple (– 0.55 V) is only slightly less negative than $MeOH/CO_2$ (– 0.64 V) and $NADH/NAD^+$ (– 0.59 V). Thus, the loss in cell potential was minimized. The catholyte consisted of platinum gauze in an O_2-saturated buffer ($O_2 + 4e^- + 4H^+ \rightarrow 2H_2O$). An open-circuit potential of 0.8 V and a maximum power output of 0.67 mW cm^{-2} was achieved.

Another biofuel cell concept is based on the oxidation of glucose to gluconolactone catalyzed by glucose oxidase (Fig. 16.2-57)[231, 232]. Because of the slow kinetics of the electron transfer to O_2, dioxygen is usually reduced at a potential several hundred millivolts more negative than its formal potential, thus lowering the power density of a fuel cell. Utilizing laccase to catalyze this reaction can circumvent that. ABTS is a suitable mediator between the electrode and laccase because of its quite positive redox potential[233]. Wiring laccase reduction to the electrode via an osmium-modified electrode also facilitates the electroreduction of molecular oxygen. The same modification serves as the conductor between glucose oxidase and the anode.

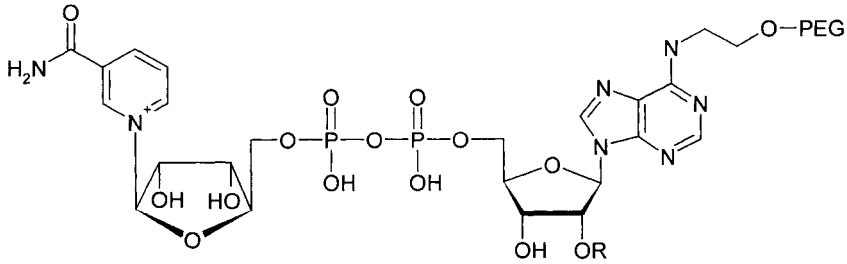


Figure 16.2-58. NAD modified with polyethylene glycol (PEG).

Table 16.2-17. Kinetic constants of different dehydrogenases for NAD(P)$^+$ and PEG-NAD(P)$^+$.

	Native cofactor	PEG-bound cofactor	
NAD$^+$-dependent enzymes	**K_M [μM]**	**K_M [μM]**	**V_{max} [as % of NAD$^+$]^a**
FDH	15	82	57
Glutamate DH	175	444	53
YADH	154	1310	64
HLADH	62	1150	72
LDH	182	142	21
3α-HSDH	29	647	66
Glucose DH	96	2030	3
NADP$^+$-dependent enzymes	**K_M [μM]**	**K_M [μM]**	**V_{max} [as % of NADP$^+$]**
Glutamate DH	160	425	96
Malic enzyme	5	12	86
TBADH	13	28	84

a 100% correspond to V_{max} values of the dehydrogenases determined with native coenzymes.

A miniaturized cell was constructed which exhibited a power output of 0.137 mW cm^{-2}. After 72 h of operation, 75% of the initial power output was still present.

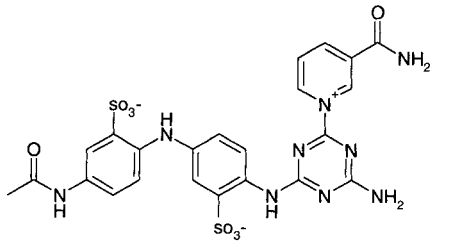
Even though biofuel cells are generally considered to be in their infancy[234], their potential, which is based on non-hazardous, easy-to-handle substrates and electrolytes (especially the moderate temperatures compared to those of conventional fuel cells: 80–1000 °C) cannot be neglected. Even photosynthetic biofuel cells (converting light energy into electrical energy) have been shown to work in principle[235].

16.2.7.2
Biomimetic Analogs to Nicotinamide Coenzymes

For large-scale applications of NAD(P)-dependent enzymes, continuous-flow reactors with ultrafiltration membranes have been proposed[236]. In order to retain low molecular weight nicotinamide cofactors in the reactor, charged membranes have been used, retarding the overall negatively charged nicotinamide coenzymes by electrostatic repulsion[237, 238]. Retention rates of approx. 99% and TTNs (NAD) of up to 10 000 were reported.

Another approach makes use of polymer-modified NAD [modification with polyethylene glycol (PEG; MW = 20 000)], thus retaining it on account of its drastically increased size (Fig. 16.2-58)[239–241]. The polymer modification usually leads to a drastically increased K_M value, whereas the V_{max} value is generally over 50% of that of low molecular weight NAD(P) (Table 16.2-17).

Another area of research deals with synthetic analogs of NAD(P) coenzymes. Besides the lower costs, these analogs may offer better stability or easier regeneration and may add new functionalities to known enzyme systems (e.g. thio-NAD together with HLADH[182]). Some artificial redox coenzymes were developed mimicking the "shape" of native nicotinamide coenzymes (Fig. 16.2-59)[242, 244]. Activity with various NAD-dependent enzymes was found, even though the activity was only



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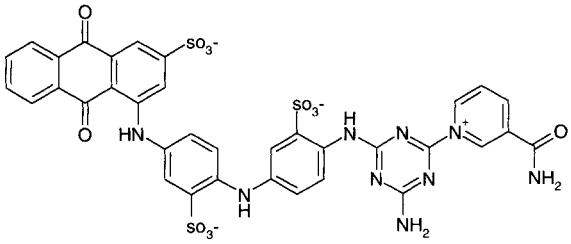

blue N-3

Figure 16.2-59. Synthetic analogs of NAD.

in the region of less than 10% of that with the native cofactor. However, it was shown that these analogs could have at least some potential.

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16.3
Oxidation of Phenols

Andreas Schmid, Frank Hollmann, and Bruno Bühler

16.3.1
Introduction

Several classes of oxidoreductases accept phenols and their derivatives as substrates for oxidation reactions. A broad range of products can be obtained depending on the substrates and enzymes applied (Fig. 16.3-1). Several monooxygenases catalyze the hydroxylation of the aromatic ring specifically *ortho* or *para* to the existing phenolic alcohol function (Fig. 16.3-1 A). Oxidases can be used to catalyze the stereospecific benzylic hydroxylation of aliphatic side chains to (*R*) or (*S*) alcohols and the further oxidation of benzylic alcohols to corresponding ketones or aldehydes; furthermore, elimination to (*Z*) or (*E*) alkenes can be obtained if desired (Fig. 16.3-1 B). Laccases and peroxidases generate phenoxy radicals which – depending on the reaction conditions – can react further with phenols to structurally complex dimers or conducting polymers (Fig. 16.3-1 C). Even nitration reactions are reported (Fig. 16.3-1 D). Thus, enzymatic modification opens up new possibilities for synthetic chemistry with aromatic compounds under mild and non-toxic conditions.

16.3.2
Oxidases

16.3.2.1
Vanillyl-alcohol oxidase (E.C. 1.1.3.38)

The enzyme vanillyl-alcohol oxidase (VAO, E.C. 1.1.3.38) was examined in detail with respect to mechanism, structural properties, and biotechnological applications by van Berkel and coworkers, giving an excellent example of how detailed biochemical studies provide a basis for preparative biocatalytic applications (for recent reviews see[1, 2]). The homooctamer with a monomer mass of 65 kDa was isolated and purified from *Penicillium simplicissimum*. The catalytic mechanism of VAO-catalyzed oxidation of *para*-alkyl phenols was studied in detail[3–5]. After initial hydride abstraction from the Cα atom, a binary complex of the intermediate *para*-quinone methide and reduced FAD reacts with molecular oxygen, regenerating the

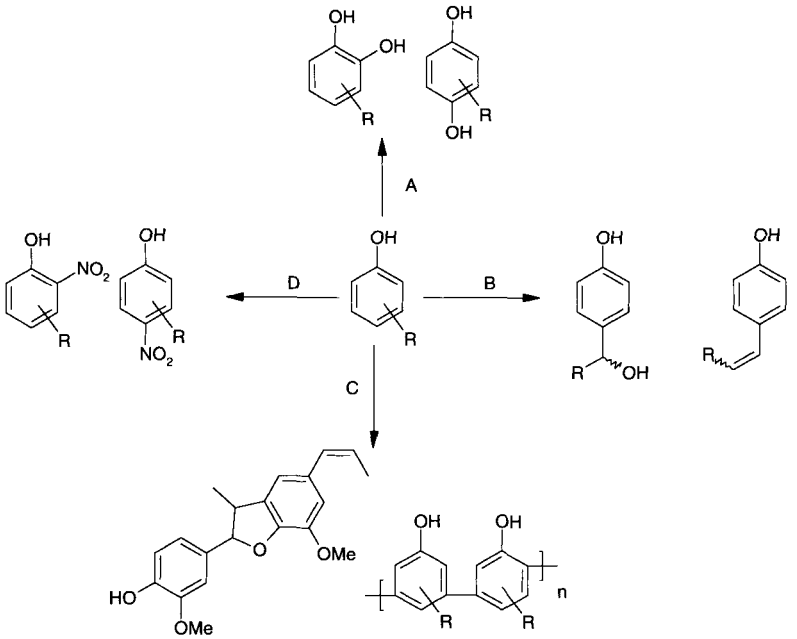


Figure 16.3-1. Enzyme-catalyzed oxidations of phenols. A: *ortho*- and *para*-hydroxylations catalyzed by monooxygenases (Sects. 16.3.3.2 and 16.3.6.2); B: oxidation at the benzylic position catalyzed by oxidases (Sects. 16.3.2.1 and 16.3.5); C: coupling reactions catalyzed by peroxidases and laccases (Sects. 16.3.4.1 and 16.3.2.2); D: nitration reactions catalyzed by peroxidases (Sect. 16.3.4.3).

oxidized prosthetic group. Depending on the nature of the aliphatic side chain, the *para*-quinone methide is hydroxylated to (chiral) benzylic alcohols (short aliphatic side chains) or rearranges yielding benzylic alkenes (long aliphatic side chains) (Fig. 16.3-2). Table 16.3-1 shows a selection of reactions catalyzed by VAO as well as the kinetic constants thereof[3, 6].

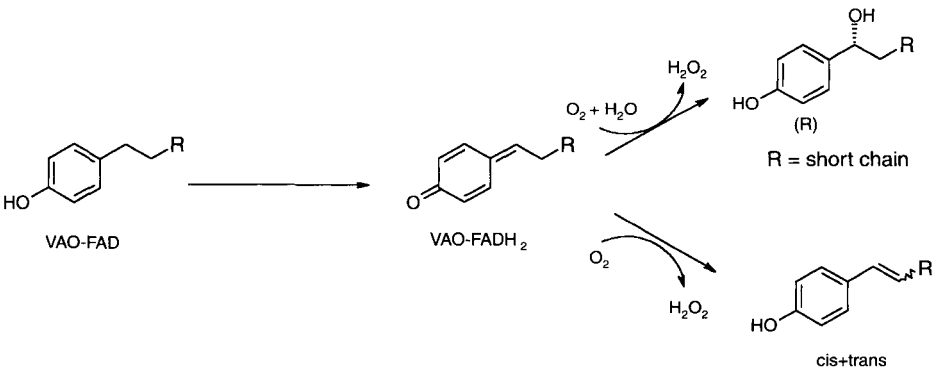


Figure 16.3-2. Reaction mechanism of vanillyl oxidase (VAO).

Table 16.3-1. Substrate spectrum and kinetic constants of vanillyl oxidase.

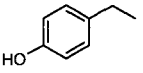
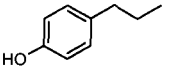
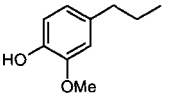
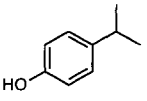
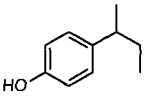
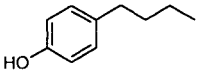
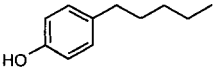
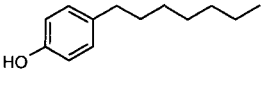
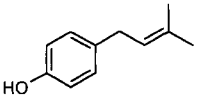
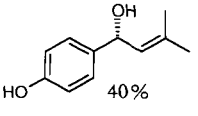
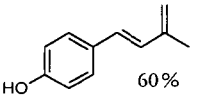
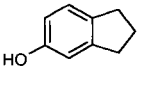
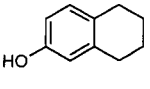
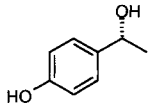
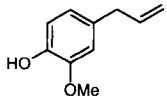
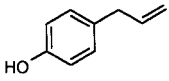
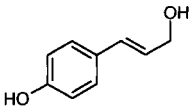
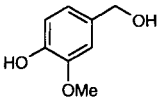
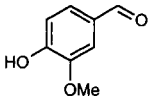
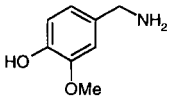
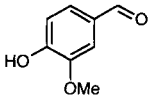
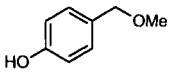
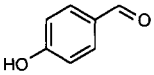
Substrate	Product(s)[a]	K_M [μM]	k_{cat} [s^{-1}]	k_{cat}/K_M [10^{-3}] [s^{-1} M^{-1}]
	76% alcohol 24% alkene	9	2.5	280
	68% alcohol 32% alkene	4	4.2	1050
	90% alcohol 10% alkene	6	4.9	820
	20% alcohol 80% alkene	16	1.3	81
	26% alcohol 74% alkene	72	0.5	7
	1% alcohol 99% alkene	2	1.2	600
	100% alkene	8	0.3	38
	100% alkene	42	< 0.001	< 0.02
	 40%  60%	65	1.4	21
	16% alcohol 60% ketone 24% alkene	77	0.5	7
	4% alcohol 2% ketone 94% alkene	94	0.7	7

Table 16.3-1. (cont.).

Substrate	Product(s)[a]	K_M [μM]	k_{cat} [s^{-1}]	k_{cat}/K_M [10^{-3}] [s^{-1} M^{-1}]
	100 % ketone	222	0.7	3
	100 % ketone	4.9	13.0	2700
		4.8	6.5	1400
		290	5.4	19
		240	1.3	5.4
		65	5.3	82

a Beside s the structure shown the products formed include benzylic alcohols, benzylic alkenes and benzylic ketones.

VAO exhibits a remarkable activity towards 4-alkylphenols, bearing aliphatic side chains of up to seven carbon atoms. The maximum chain-length of 7 is in accordance with structural data obtained from X-ray crystallography[7]. Short-chain 4-alkylphenols are mainly hydroxylated at the Cα position, whereas medium-chain 4-alkylphenols are dehydrogenated to 1-(4'-hydroxyphenyl)alkenes (Fig. 16.3-2)[6]. The hydroxylation reaction is highly stereospecific, producing the (*R*)-enantiomer with *ee* values of up to 94 %[8]. Furthermore, VAO also catalyzes the further oxidation of the alcohols to the corresponding ketones. Here, the VAO-catalyzed oxidation of (*S*)-alcohols is far more efficient than the oxidation of (*R*)-alcohols, promoting a possible application in kinetic resolution reactions. Substrates with more space-consuming alkyl side chains are dehydrogenated by the action of VAO. With *para*-methyl phenols (e.g. cresol), a very low conversion rate is found which is due to the formation of a stable intermediate formed through a nucleophilic attack of the reduced FAD on the *para*-quinone methide, yielding a covalent bond[2]. Since the rate-limiting hydrolysis of this intermediate is acid-catalyzed, the pH optimum of the reaction shifts from alkaline to acidic values. The formation of such a covalent

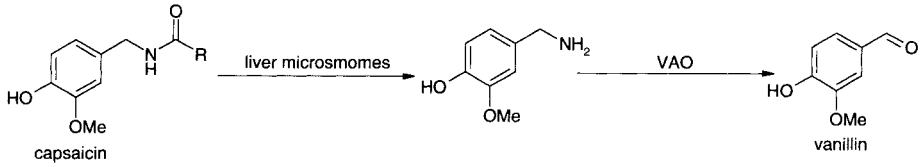


Figure 16.3-3. Potential biotechnological production route to vanillin from natural components with vanillyl oxidase.

intermediate is supposed to be more unlikely with increasing length of the aliphatic side chain, because of increasing steric hindrance.

Much attention has been paid to the shift from hydroxylation to dehydrogenation with increasing length of the side chain. The product ratio between alcohols and alkenes is strongly influenced by the extent of hydratation of the intermediate, *para*-quinone methide. Thus, by using organic media with a low water content the overall alkene yield could be significantly increased. The same is true for monovalent anions such as Cl^-, Br^-, or SCN^-, which bind to the active site, thereby decreasing the water concentration at the active site[9]. By enzyme engineering based on the three-dimensional structure[7], the ratio between hydroxylation products and dehydrogenation products could be shifted either in favor of the alcohols, when Asp170 was exchanged with Glu, or in favor of the alkenes, when Asp170 was exchanged with Ser[10]. Double mutants of VAO (D170S/T457E and D170A/T457E) were produced based on the same rational approach, thus inverting the stereospecificity of the VAO-catalyzed hydroxylation of 4-ethyl phenol from (*R*) to (*S*) (*ee* = 80%)[11].

The VAO-catalyzed production of vanillin is of special synthetic interest. In particular, a route starting from capsaicin that is readily available from red hot pepper has some biotechnological potential. Here, vanillylamine is obtained by hydrolysis of capsaicin using rat liver microsomes and further oxidized by VAO (Fig. 16.3-3). Furthermore, a one-pot synthesis using carboxylesterase for capsaicin hydrolysis is proposed[12].

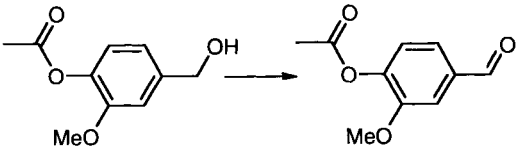
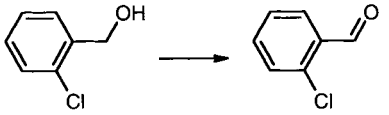
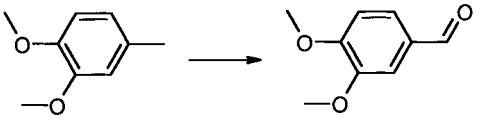
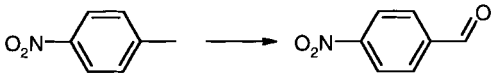
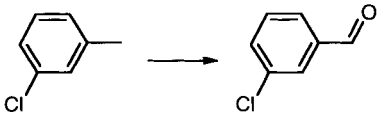
16.3.2.2
Laccase (E.C. 1.10.3.2)

Recently, laccases found some interest for synthetic application. Laccases are widely distributed in plants and fungi[13]. The copper-containing enzymes are some of the few oxidases so far reported to reduce molecular oxygen to water (aside from cytochrome c oxidase and others). This ability was recently exploited in a novel regeneration concept for flavin-dependent enzymes (see Chapter 16.2)[14].

Purified laccase oxidizes various phenolic compounds via hydrogen abstraction. The resulting phenoxy radical undergoes various dimerization and oligomerization reactions. Even though the synthetic potential of such reactions has to be considered as moderate, in some cases interesting products (such as complex coumaran type compounds) can be obtained in reasonable yields from simple phenols[15].

Laccases alone are not able to oxidize benzyl alcohols. Bourbonnais and Paice[16]

Table 16.3-2. Laccase/ABTS-catalyzed oxidations to corresponding aldehydes.

Catalyzed reaction	Yield [%]	Literature
	94	[1]
	92	[1]
	92	[1]
	98	[1]
	90	[1]
	92	[2]
	98	[2]
	89	[2]

1 A. Potthast, T. Rosenau, C. L. Chen, J. S. Gratzl, *J. Mol. Cat. A.: Chemical* **1996**, *108*, 5–9.

2 A. Potthast, T. Rosenau, C. L. Chen, J. S. Gratzl, *J. Org. Chem.* **1995**, *60*, 4320–4321.

were the first to report that laccase in the presence of a specific compound, usually called a "mediator", is able to catalyze the oxidation of benzyl alcohols. Mostly ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid), HOBT (1-hydroxybenzotriazole) [17], and NHAA (*N*-hydroxyacetanilide) [18] have been used as mediators so far.

The actual role of the mediator is not yet fully understood, although Potthast *et al.* recently found evidence that laccase produces reactive radical species of ABTS and

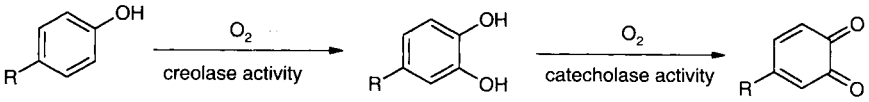


Figure 16.3-4. Oxidation of phenols catalyzed by tyrosinase displaying so-called creolase and catecholase activities.

HOBT, which perform the actual oxidations[17]. Nevertheless, some preparative oxidations of various benzylic alcohols are reported (Table 16.3-2).

It should be pointed out here that the laccase-mediator system still is far from being economically feasible.

16.3.3
Monooxygenases

16.3.3.1
Tyrosinase (E.C. 1.10.3.1)

Tyrosinases (synonyms: phenol oxidases, poly-phenolases or polyphenol oxidases) are copper-containing monooxygenases, which catalyze two consecutive reactions with molecular oxygen as cosubstrate, namely the *ortho*-hydroxylation of phenols and the oxidation of the resulting catechols to *ortho*-quinones (Fig. 16.3-4).

The initial (phenol-hydroxylating) activity is usually referred to as creolase activity, whereas the second (catechol-oxidizing) activity is most commonly called catecholase activity[19]. The classification of tyrosinases (polyphenol oxidases) is somewhat ambiguous; enzymes exhibiting monophenol oxidase activity are classified as E.C. 1.14.18.1., but those with catechol oxidase activity as E.C. 1.10.3.2. However, many enzymes exhibit both activities, and a more appropriate classification of all two-electron-accepting copper monooxygenases as E.C. 1.14.18.1 was proposed[20].

In animals, tyrosinase is involved in the formation of melamines, and in plants, tyrosinase leads to the well-known browning of open surfaces of fruits[21].

Much attention has been paid to the mechanism[20, 22]. In the active site, two copper(I) ions bind molecular oxygen. Upon binding of the phenolic substrate, the *ortho*-position is attacked electrophilically by one of the activated oxygen atoms. The resulting copper-bound catechol serves as an internal electron donor and leaves the active site as *ortho*-quinone. Figure 16.3-5 illustrates this mechanism.

In order to prevent rapid quinone polymerization in aqueous media, the quinones are usually reduced to the catechols (most commonly by ascorbic acid) (Fig. 16.3-6).

Several tyrosinase-catalyzed oxidations of phenols have been reported; some of these are presented in Table 16.3-3.

Tyrosinase was reported to hydroxylate and oxidize tyrosine residues in proteins[23], which is important in the production of moisture-resistant adhesives. In fact, tyrosinase has been used for the production of synthetic glues with similar compositions to those of naturally occurring adhesives such as mussel glue[24].

An interesting cascade reaction was reported by Waldmann *et al.*[25, 26]. Tyr-

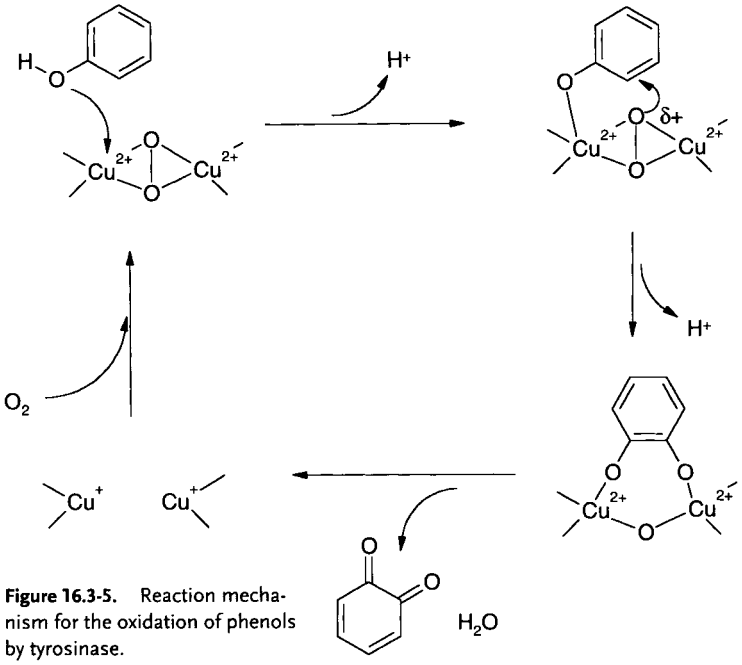


Figure 16.3-5. Reaction mechanism for the oxidation of phenols by tyrosinase.

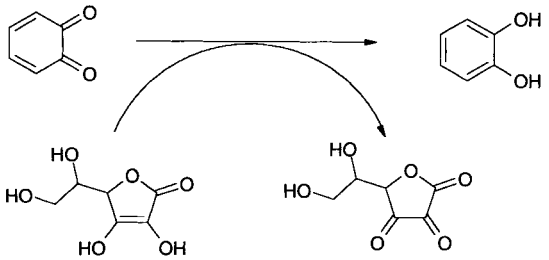


Figure 16.3-6. Ascorbic acid-driven reduction of quinones.

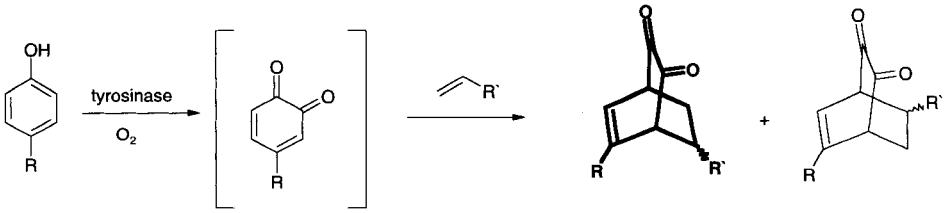
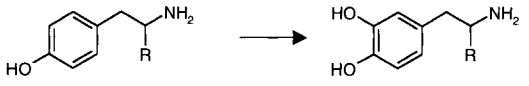
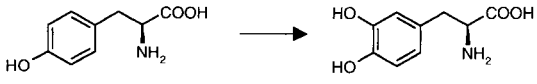
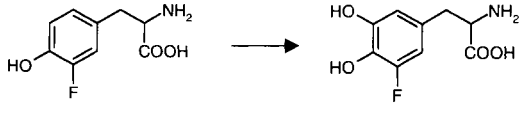
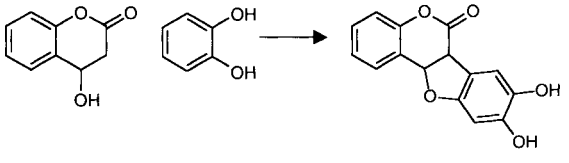
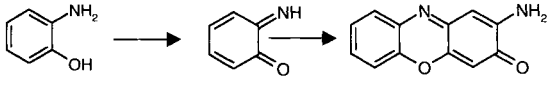


Figure 16.3-7. Chemoenzymatic Diels-Alder reactions. *Ortho*-quinones (dienes), derived from phenols by oxidation with tyrosinase, spontaneously react with dienophils.

osinase, immobilized on glass beads, was used to oxidize several phenols in chloroform as the organic medium. The products of the enzymatic oxidation step, the *ortho*-quinones, served *in situ* as dienes in a Diels-Alder reaction (Fig. 16.3-7). Table 16.3-4 summarizes some phenols (dienes after enzymatic oxidation) and dienophiles with which such a reaction cascade was observed.

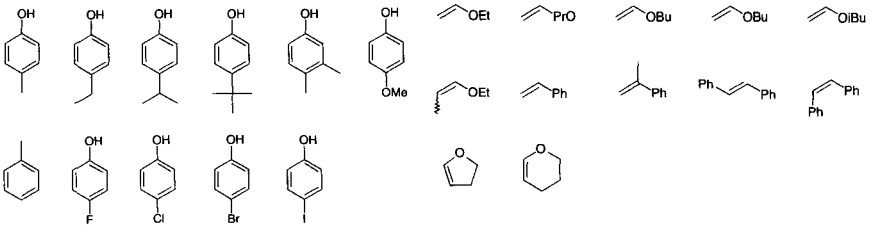
Table 16.3-3. Oxidations of phenols catalyzed by tyrosinase.

Substrate	Product	References and remarks
R = OCH_3, OC_2H_5, CH_3, $C(CH_3)_3$, Halogen, etc.		Electron-rich phenols are preferred[3]
 R = H, CH_3		[4, 5]
		L-DOPA production[6]
		Possible agent in melanoma treatment[7, 8]
		Coumestans[9]
		Phenoxazones[10]

3 S. Passi, M. Nazzaro-Porro, *Brit. J. Dermatol.* **1981**, *104*, 659.
4 M. Jimenez, F. Garcia-Carmona, F. Garcia-Canovas, J. L. Iborra, J. A. Lozano, F. Martinez, *Arch. Biochem. Biophys.* **1984**, *235*, 438.
5 M. Jimenez, F. Garcia-Carmona, F. Garcia-Canovas, J. L. Iborra, J. A. Lozano, *Int. J. Biochem.* **1985**, *17*, 891.
6 G. M. Carvalho, T. L. M. Alves, D. M. G. Freire, *Appl. Biochem. Biotech.* **2000**, *84–86*, 791–800.
7 M. E. Rice, B. Moghaddam, C. R. Creveling, K. R. Kirk, *Anal. Chem.* **1987**, *59*, 1534.
8 R. S. Phillips, J. G. Fletscher, R. L. Von Tersch, K. L. Kirk, *Arch. Biochem. Biophys.* **1990**, *276*, 65.
9 U. T. Bhalearo, C. Muralikrishna, G. Pandey, *Synth. Commun.* **1989**, *19*, 1303.
10 O. Toussaint, K. Lerch, *Biochem.* **1987**, *26*, 8567.

By this reaction sequence, highly functionalized bicyclo-[2.2.2]-octenes can be obtained from simple phenols and alkenes as starting materials. The overall yields reported are usually satisfactory (> 70%). The Diels-Alder products are racemic, probably because the Diels-Alder reaction proceeds in the bulk organic phase without involvement of tyrosinase.

Table 16.3-4. Substrates for the reaction cascade including tyrosinase catalyzed oxidation of phenols and a Diels-Alder-reaction[11, 12].

Phenols	Dienophiles
	

Via a tyrosinase catalyzed reaction the phenols are transformed to dienes, which subsequently react with the dienophiles in a Diels-Alder-reaction as shown in Figure 16.3-7.

11 G. H. Müller, H. Waldmann, *Tetrahedron Lett.* 1996, *37*, 3833–3836.

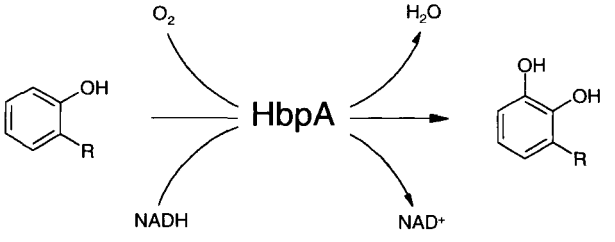
12 G. H. Müller, A. Lang, D. R. Seithel, H. Waldmann, *Chem. Eur. J.* 1998, *4*, 2513–2522.

16.3.3.2
2-Hydroxybiphenyl-3-monooxygenase (HbpA, E. C. 1.14.13.44)

The flavin-dependent, homotetrameric HbpA is the first enzyme in the biodegradation pathway of 2-hydroxybiphenyl in *Pseudomonas azelaica* HBP1[27]. HbpA catalyzes the selective *ortho*-hydroxylation of a broad range of phenols to the corresponding catechols, utilizing NADH as cofactor (Fig. 16.3-8 and Table 16.3-5).

Compared to the chemical synthesis of *ortho*-substituted catechols (*ortho*-hydroxylation and aromatization procedures)[28–31], such an enzymatic approach is superior with respect to the number of steps involved as well as simplicity, selectivity, and yield. The resulting *ortho*-substituted catechols are valuable building blocks[32].

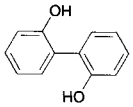
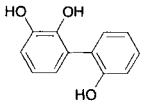
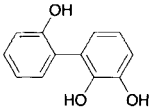
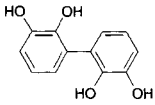
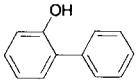
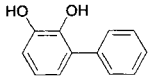
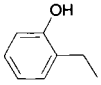
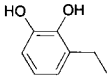
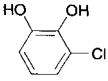
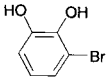
HbpA is an excellent example of *in vivo* as well as *in vitro* biocatalysis. Since the desired catechols are rapidly degraded via the *P. azelaica meta*-cleavage pathway by two catechol-2,3-dioxygenases, the gene coding for HbpA was expressed in *E. coli* JM109, which served as a biocatalyst accumulating the desired products[33]. Drawbacks such as inhibition by substrate and product can be overcome by continuous substrate feeding and *in situ* recovery of the catechol products with solid adsorbents



R = Ph, 2'-OH-Ph, 2,3-$(OH)_2$Ph, F, Cl, Br, Me, Et, Pr, *i*-Pr, But

Figure 16.3-8. Reaction scheme for the *ortho*-hydroxylation of phenol derivatives catalyzed by 2-hydroxybiphenyl-3-monooxygenase (HbpA).

Table 16.3-5. Substrates and relative activities of 2-hydroxybiphenyl-3-monooxygenase (HbpA)[13].

Substrate	Product	Relative activity [%][a]
		100
		34
		49 Native substrate
		24
		36
		10
		20
		33

a Relative activities were determined polarographically with whole cells of recombinant *E. coli* containing HbpA. 100% corresponds to the HbpA-dependent specific oxygen uptake rate of whole cells incubated with 2,2'-dihydroxybiphenyl.

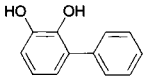
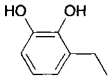
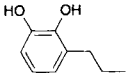
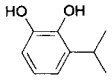
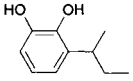
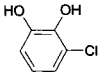
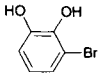
13 A. Schmid, H.-P. E. Kohler, K.-H. Engesser, *J. Mol. Cat. B: Enzymatic* **1998**, *5*, 311–316.

in such a way that substrate and product concentrations can be kept below toxic levels[32]. Thus, several 3-substituted catechols were produced in gram amounts with satisfactory to high yields (Table 16.3-6).

The *in vivo* processes are based on a recombinant *E. coli* as catalyst[33]. Optimized space-time yields of up to 0.39 g L^{-1} h^{-1} for the formation of 3-phenyl catechol from 2-phenyl phenol can be reached[34].

The enzyme itself was purified and characterized in detail[27, 35]. Based on this knowledge and via directed evolution, HbpA characteristics were modified (Meyer, Schmid and Witholt, unpublished results) yielding HbpA variants with improved

Table 16.3-6. Preparative-scale production of 3-substituted catechols using *E. coli* JM101 containing 2-hydroxybiphenyl-3-monooxygenase[14].

Product	Product recovered [g]	Molar yield [%]
	8.1	94
	2.1	95
	0.6	71
	2.2	77
	1.7	85
	0.9	71
	2.1	71

14 M. Held, W. Suske, A. Schmid, K. Engesser, H. Kohler, B. Witholt, M. Wubbolts, *J. Mol. Cat. B: Enzymatic* **1998**, *5*, 87–93.

catalytic properties and changed substrate spectrum. For example, a new mutant with drastically decreased unproductive NADH oxidation and concomitant formation of hydrogen peroxide was developed. This so-called uncoupling reaction is quite common amongst flavin-dependent monooxygenases, and represents the major mechanism of autoregeneration amongst oxidases. Furthermore, the activity toward several substrates that are poorly converted by native HbpA, such as 2-*sec*-butylphenol (30% activity increase), 2-*tert*-butylphenol (fivefold activity increase) or guaiacol (more than eightfold increase in K_M/k_{cat}), could be improved[36]. The HbpA substrate spectrum could be enlarged even more via directed evolution. Recently, an HbpA mutant was found that initiated the production of indigo starting from indole. It is assumed that HbpA converts indole into the 2,3-epoxide, which spontaneously dimerizes to indigo (Fig. 16.3-9)[37].

In vitro application of HbpA (and monooxygenases in general) offers some advantages over whole-cell biotransformations. For example, toxic effects on cell metabolism and further metabolization of the desired product can be avoided, and experimentally demanding *in vivo* set-ups are not necessary (beneficial for organic chemists). The major challenge in *in vitro* biotransformations is the efficient

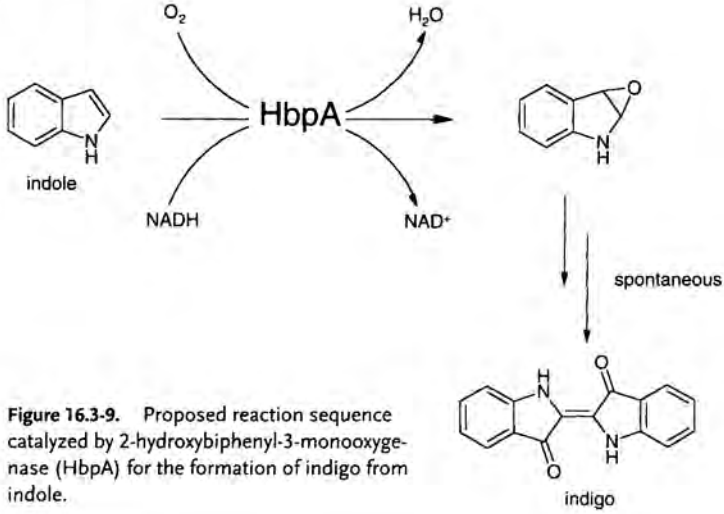


Figure 16.3-9. Proposed reaction sequence catalyzed by 2-hydroxybiphenyl-3-monooxygenase (HbpA) for the formation of indigo from indole.

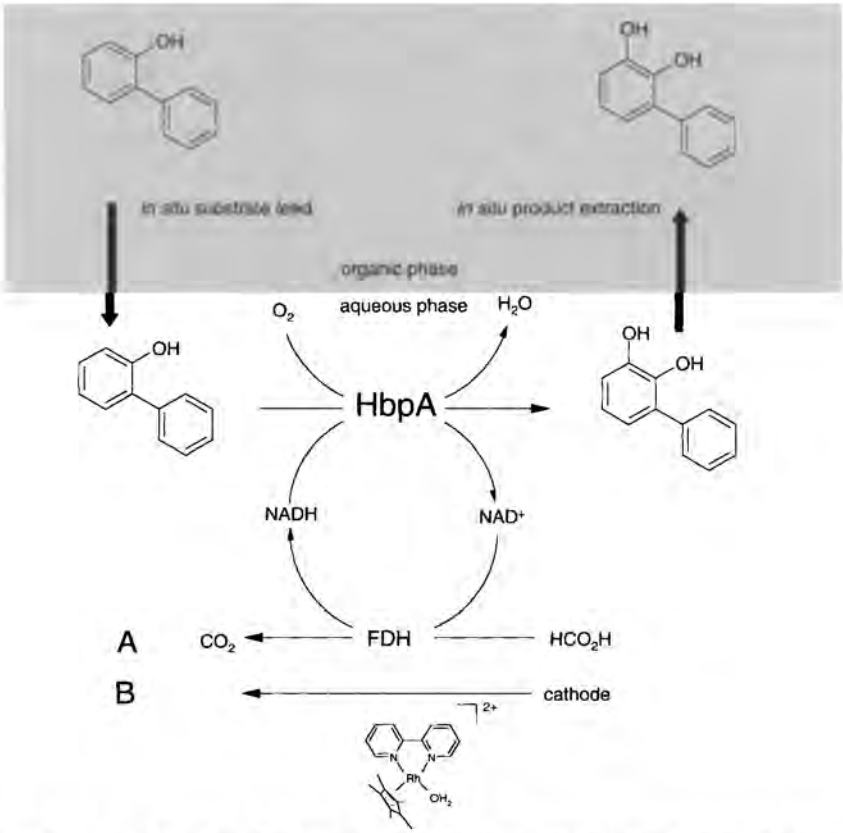
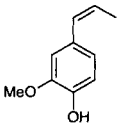
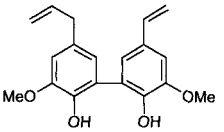
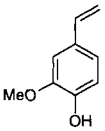
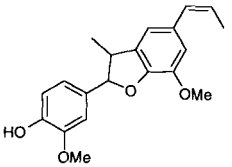
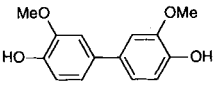
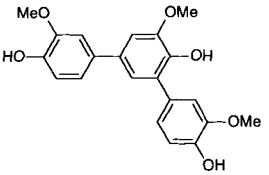
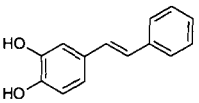
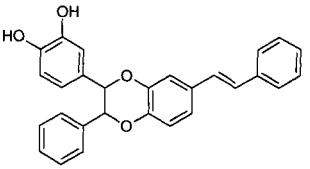
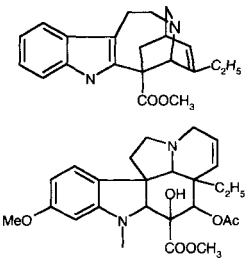
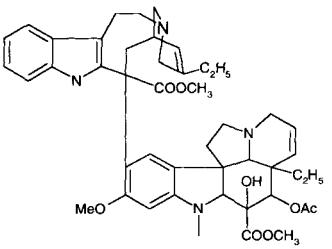


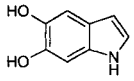
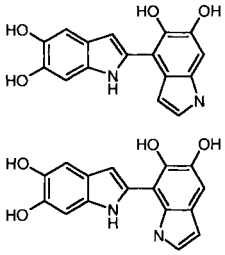
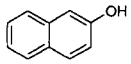
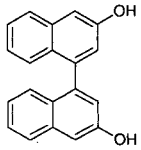
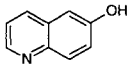
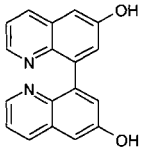
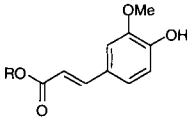
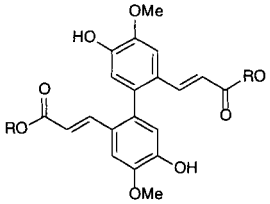
Figure 16.3-10. Formation of 3-phenylcatechol from 2-phenylphenol catalyzed by partially purified 2-hydroxybiphenyl-3-monooxygenase (HbpA) in organic aqueous emulsions. Regeneration of NADH was achieved *in situ* with formate dehydrogenase (FDH) (A) or indirectly electrochemically with $[Cp^*Rh(bpy)(H_2O)]^{2+}$ (B).

Table 16.3-7. Substrates and products of peroxidase – catalyzed oxidative di- and oligomerizations of phenols.

Substrate	Products	References and applications
		Alkaloid synthesis[15]
		Alkaloid synthesis[15]
		
		Antimicrobial compounds[16]
		Phytoalexin activity[17]
		Cancer theraphy[18]

regeneration of reduced nicotinamide coenzymes. The general strategies are described in Chapter 7. Furthermore, the production enzyme must be easily available in large amounts. HbpA was obtained in gram amounts from recombinant *E. coli* in a one-step operation via expanded bed adsorption chromatography[38]. Limitations

Table 16.3-7. (cont.).

Substrate	Products	References and applications
		Melanin synthesis[19]
		Racemic[20]
		Racemic[20]
		Quest Int. Naarden, The Netherlands, R = arrabinoxylan, carbohydrate gel which retains water

15 A. R. Krawczyk, E. Lipkowska, J. T. Wrobel, *Coll. Czech. Chem. Commun.* **1991**, *56*, 1147.
16 A. Kobayashi, Y. Koguchi, H. Kanzaki, S. I. Kajiyama, K. Kawazu, *Biosci. Biotech. Biochem.* **1994**, *58*, 133.
17 D. M. X. Donelly, F. G. Murphy, J. Polonski, T. Prangé, *J. Chem. Soc. Perkin Trans. I* **1987**, 2719.
18 A. E. Goodbody, T. Endo, J. Vukovic, J. P. Kutney, L. S. L. Choi, M. Misawa, *Planta Med.* **1988**, 136.
19 M. d'Ischia, A. Napolitano, K. Tsiakas, G. Prota, *Tetrahedron* **1990**, *46*, 5789.
20 M. M. Schmitt, E. Schüler, M. Braun, D. Häring, P. Schreier, *Tetrahedron Lett.* **1998**, *39*, 2945–2946.

due to low solubility of substrates and products can be overcome in biphasic reaction systems (Fig. 16.3-10). HbpA exhibits significant activity in the presence of various organic solvents such as 1-decanol, hexadecane or heptane[39].

Thus, the synthetic *in vitro* application of HbpA was done via an emulsion process. Several regeneration strategies for NADH were reported (Fig. 16.3-10).

In the emulsion process, a high 3-phenylcatechol concentration in the organic phase and the same or higher productivities (up to 0.45 g L^{-1} h^{-1}) as in the *in vivo* process were achieved[40]. Here, formate dehydrogenase and formate served as the coenzyme regeneration system (Fig. 16.3-10 A). The benefits of this regeneration

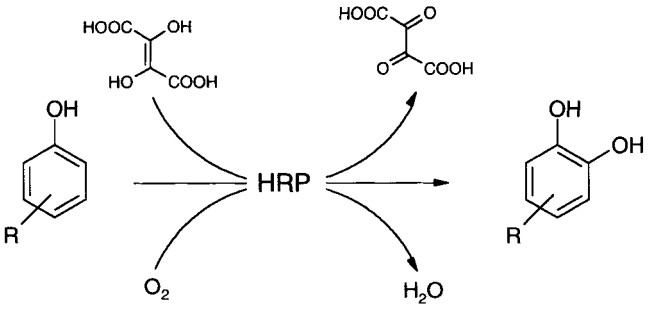


Figure 16.3-11. Hydroxylation of phenols to catechols catalyzed by horseradish peroxidase (HRP).

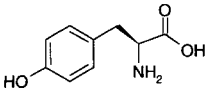
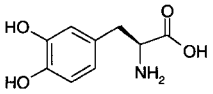
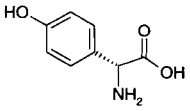
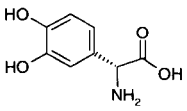
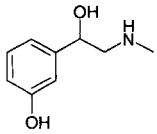
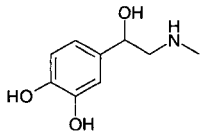
system are described in Chapter 16.6. Even electrical power could be used as a source of reduction equivalents (Fig. 16.3-10 B)[41].

16.3.4
Peroxidases

16.3.4.1
Oxidative Coupling Reactions

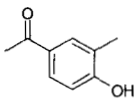
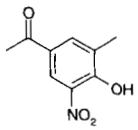
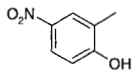
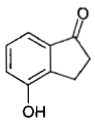
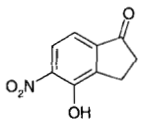
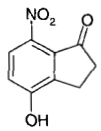
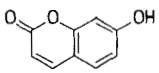
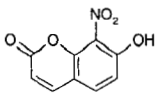
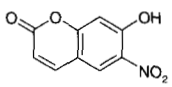
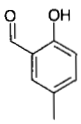
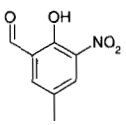
Phenols are typical substrates for peroxidases. Quite similarly to the laccase-mechanism (described earlier in this chapter), peroxidases catalyze phenol oxidations via hydrogen abstraction. The radicals thus generated leave the active site and

Table 16.3-8. Selected hydroxylation reactions of phenols catalyzed by horseradish peroxidase.

Substrate	Product	Literature
 Tyrosine	 L-Dopa	[21]
		[21]
	 Adrenaline	[21]

21 A.M. Klibanov, Z. Berman, B.N. Alberti, *J. Am. Chem. Soc.* **1981**, *103*, 6263–6364.

Table 16.3-9. Selected nitration reactions of phenols catalyzed by soybean peroxidase.

Substrate	Product(s), Yield [%] *ortho*	*para*
	 58	 27
	 22	 25
	 41	 20
	 25	–

react with other aromatic compounds (depending on the reaction conditions) to form dimeric and polymeric products[42]. A selection of dimeric products is presented in Table 16.3-7.

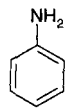
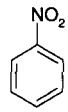
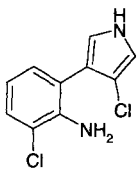
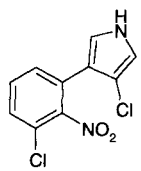
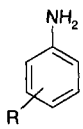
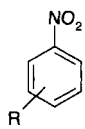
Recently, peroxidases, especially horseradish (HRP) and soybean peroxidase, found increasing interest in resin manufacturing. The peroxidase-catalyzed coupling of phenols[43], catechols[44], hydroquinones[45], or anilines[46, 47] is a potential substitute for the conventional production of phenolic resins using toxic formaldehyde[48]. The resins find applications as conductive polymers[45, 49].

16.3.4.2
Hydroxylation of Phenols

As early as 1961, Mason and coworkers reported that HRP, in the presence of dihydrofumaric acid as cofactor, catalyzes the hydroxylation of arenes (Fig. 16.3-11)[50].

Also lignin peroxidase was found to catalyze the oxidation of phenol, cresol, and tyrosine[51].

Table 16.3-10. Oxidation reactions of arylamines catalyzed by peroxidases.

Substrate	Product	References and remarks
		Bromoperoxidase[22]
 Aminopyrrolonitrin	 Pyrrolonitrin	Chloroperoxidase[23]
		Chloroperoxidase[24] R = *o*-, *m*-, *p*-Cl; *p*-CH_3; *p*-COOH

22 N. Itoh, N. Morinaga, T. Kouzai, *Biochem. Mol. Biol.* **1993**, *29*, 785–791.
23 S. Kirner, K.-H. van Pee, *Angew. Chem. Int. Ed.* **1994**, *33*, 352.
24 V. N. Burd, K.-H. van Pee, *Bioorg. Khim.* **1998**, *24*, 462–464.

16.3.4.3
Nitration of Phenols

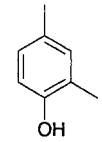
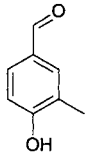
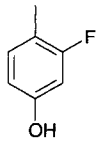
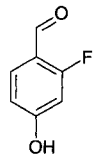
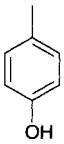
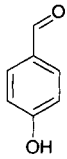
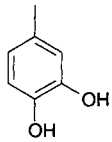
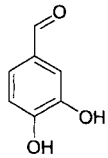
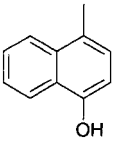
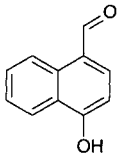
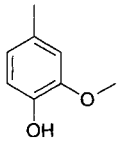
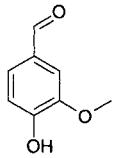
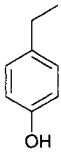
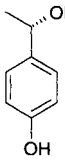
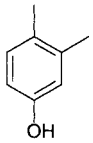
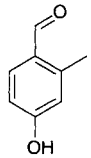
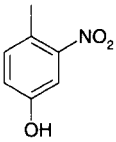
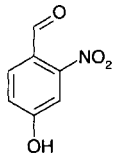
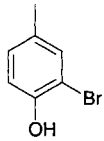
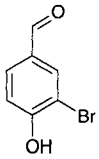
Khmelnitsky and coworkers recently reported a rather unusual application of soybean peroxidase. In the presence of nitrite and hydrogen peroxide, phenols are nitrated. The nitration of tyrosine has been reported earlier[52, 53]. The substrate spectrum was enlarged by various phenolic compounds (Table 16.3-9). Thus, such an enzymatic nitration represents an alternative to chemical nitration (especially for acid-labile phenols, which cannot by nitrated chemically).

Other peroxidases such as HRP or CPO were also able to perform such reactions.

Another approach to the production of nitroarenes with peroxidases is based on the CPO (or bromoperoxidase)-catalyzed oxidation of arylamines. Table 16.3-10 gives a selection of peroxidase-catalyzed conversions of aniline derivatives to corresponding nitroarenes.

For example, aniline was converted into nitrobenzene by a bromoperoxidase from *Pseudomonas putida*[54], and aminopyrrolonitrin was converted into the antibiotic pyrrolonitrin by a CPO from *P. pyrrocinia*[55].

Table 16.3-11. Substrates and products of 4-cresol-oxidoreductase[25, 26].

Substrate	Product	Substrate	Product
			
			
			
			
			

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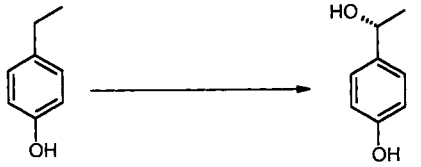
16.3.5
Other Oxidoreductases

16.3.5.1
4-Cresol-oxidoreductase (PCMH, E. C. 1.17.99.1)

This enzyme shares structural and mechanistic properties with VAO[11]. In contrast to VAO it is not an oxidase as regeneration of the covalently bound FAD with molecular oxygen is not possible. It is a flavocytochrome enzyme. The reduction equivalents from the substrate are transferred to a type c cytochrome[56, 57]. In

Table 16.3-12. Oxidations of 4-alkylphenols catalyzed by 4-ethylphenol oxidoreductase[27].



> 98% *e. e.*

Substrate	Relative conversion rate [%][a]
p-Cresol	44
4-Ethylphenol	100
4-Propylphenol	112
4-Butylphenol	114
4-Pentylphenol	116
4-Heptylphenol	52
4-Nonylphenol	14

a 100% corresponds to the 4-ethylphenol conversion rate.

27 C. D. Reeve, M. A. Carver, D. J. Hopper, *Biochem. J.* **1990**, *269*, 815–819.

addition to a cytochrome c / cytochrome c oxidase regeneration system[58], chemical reoxidation agents such as phenazine methosulfate, dichlorophenol indophenol[59], and ferrocenes[60–62] have been used.

The reaction mechanism is quite similar to the one of VAO and also includes an intermediate, the *para*-quinone methide. Like VAO, 4-cresol-oxidoreductase also exhibits a high enantioselectivity for (*S*)-1-(4'-hydroxyphenyl)alkylalcohols[59].

This enzyme accepts a broad range of substrates; *para*-methylphenols are preferably oxidized to the corresponding aldehydes, whereas the oxidation of *para*-alkylphenols results in the formation of significant amounts of (*S*)-alcohols (Table 16.3-11)[59, 63].

16.3.5.2
4-Ethylphenol Oxidoreductase

4-Ethylphenol oxidoreductase from *Pseudomonas putida* JD1 is structurally almost identical to 4-cresol oxidoreductase, but catalyzes the hydroxylation of *para*-alkylphenols with longer aliphatic chains (Table 16.3-12). The hydroxylation reactions enantioselectively produce (*R*)-alcohols[64, 65]. The regeneration properties of this enzyme are quite similar to 4-cresol oxidoreductase[61].

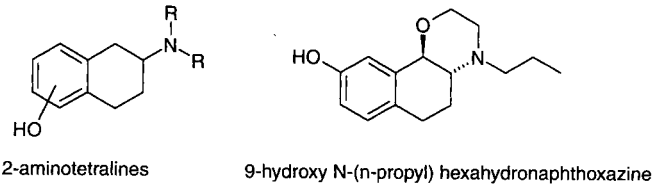


Figure 16.3-12. Substrates for phenol oxidase from *Mucuna pruriens*. 5-, 6-, or 7-Hydroxylated 2-aminotetralins with R = H or C_3H_7 and 9-hydroxy-*N*-(*n*-propyl)-hexahydronaphthoxazine are substrates for the phenol oxidase.

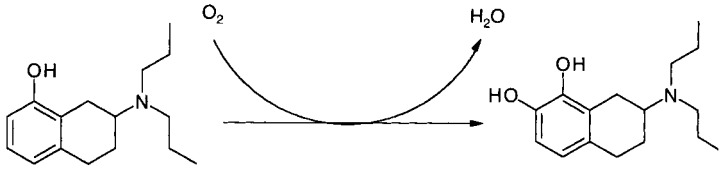


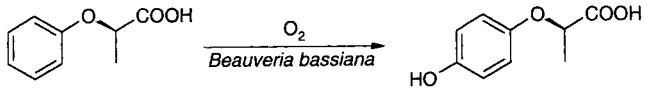
Figure 16.3-13. Formation of 7,8-dihydroxy *N*-(di-*n*-propyl)-2-aminotetralin with *Mucuna*-phenoloxidase. Quinone formation is prevented *in situ* with ascorbate as reductant.

16.3.6
In vivo Oxidations

16.3.6.1
Phenoloxidase of *Mucuna pruriens*

Like other phenoloxidases, this enzyme has a low substrate specificity and is able to *ortho*-hydroxylate a whole range of *para*-substituted monocyclic phenols. The catechols produced belong to groups of fine chemicals and pharmaceuticals[66]. Furthermore, also bi- and tri-cyclic phenols were converted into catechols (Figure16.3-12)[67]. 2-Aminotetralines, on the basis of their dopaminergic properties, are compounds of pharmaceutical interest.

Phenoloxidase (monophenol monooxygenase, E.C. 1.14.18.1) introduces one atom of molecular oxygen into the substrate and was used in alginate-entrapped cells or in partially purified form. The pharmaceutical 7,8-dihydroxy-*N*-(di-*n*-propyl)-2-aminotetralin was produced continuously using a phenol oxidase suspension in dialysis tubing in an airlift fermenter coupled to an aluminium oxide column for selective product isolation (Figure 16.3-13)[68]. A product concentration of 130 mg/L and a yield of 25 % were reached.



(*R*)-2-phenoxypropionic acid (*R*)-2-(4-hydroxyphenoxy)propionic acid

Figure 16.3-14. Regioselective *para*-hydroxylation of (*R*)-2-phenoxypropionic acid catalyzed by *Beauveria bassiana* (HPOPS process).

16.3.6.2
Monohydroxylation of (R)-2-Phenoxypropionic Acid and Similar Substrates [69, 70]

The product is a frequently used intermediate for the synthesis of enantiomerically pure aryloxyphenoxypropionic acid type herbicides. The enzyme catalyzing the hydroxylation of the phenolether is an oxidase, which is not further characterized. The biocatalyst *Beauveria bassiana* was found by an extensive screening of microorganisms for regioselective hydroxylation of (*R*)-2-phenoxypropionic acid and for substrate tolerance. This fungal strain was improved by random mutagenesis and screening, which resulted in strain LU 700. The hydroxylation is not growth-associated and the *ee* is increased during oxidation from 96% for the substrate to 98% for the product. After process optimization, a productivity of 7 g L^{-1} d^{-1} was reached. The biotransformation is carried out in a 120 000 L reactor at BASF in Germany.

The biocatalyst has a broad substrate spectrum. A compound needs the structural elements of a carboxylic acid and an aromatic ring system to be a substrate for the oxidase. Hydroxylation primarily takes place at the *para* position if it is free. If an alkyl group is in the *para* position, only the side chain is oxidized. In systems with more than one ring, the most electron-rich ring is hydroxylated.

16.3.6.3
Biotransformation of Eugenol to Vanillin [71]

The biotechnological production of vanillin is of interest because there is a large demand for vanillin originating from so called "natural" sources. Possible strategies for the biotechnological production of vanillin are reviewed by Priefert *et al.* [72].

One synthetically interesting strategy is the production of vanillin from eugenol. Here, a part of a catabolic pathway is used to accumulate an intermediate of this pathway. This was achieved by the knock-out of the enzyme catalyzing the further conversion of the putative product.

For the accumulation of vanillin from eugenol, the catabolism of eugenol in *Pseudomonas* sp. Strain HR199 (DSM7063) was used. In order to prevent further degradation of vanillin, the gene enconding vanillin dehydrogenase, responsible for the oxidation of vanillin to vanillic acid, was inactivated by insertion mutagenesis.

In a non-optimized biotransformation using growing cells in an aqueous mineral salts medium containing gluconate as a source of carbon and energy and 6.5 mM eugenol, vanillin accumulated up to a concentration of 2.9 mM, corresponding to a

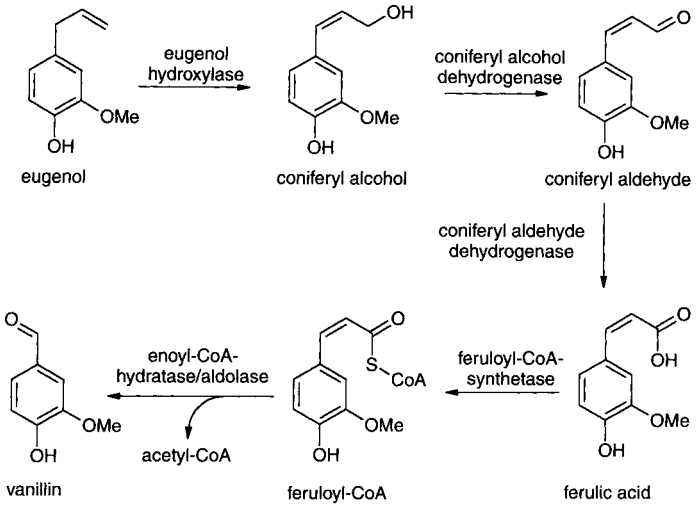


Figure 16.3-15. Multistep biotransformation of eugenol to vanillin catalyzed by whole cells of *Pseudomonas* sp. HR 199.

molar yield of 44.6%. The major drawback of the process is the degradation of vanillin by the action of coniferyl aldehyde dehydrogenase when coniferyl aldehyde is depleted from the medium.

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72 H. Priefert, J. Rabenhorst, A. Steinbüchel, *Appl. Microbiol. Biotech.* **2001**, *56*, 296–314.

16.4
Oxidation of Aldehydes

Andreas Schmid, Frank Hollmann, and Bruno Bühler

16.4.1
Introduction

To date, few reports on synthetic enzymatic oxidations of aldehydes have been published. Preparative applications reported include bioconversions of natural products such as retinal (Fig. 16.4-1 A) and various aliphatic and unsaturated aldehydes (Fig. 16.4-1 B). A broad range of aromatic acids can be obtained from their corresponding aldehydes (Fig. 16.4-1 C). Another reported reaction type is the production of olefins from aldehydes by oxidative removal of formic acid from the substrate (Fig. 16.4-1 D).

16.4.2
Alcohol Dehydrogenases

Alcohol dehydrogenases are generally applied for the interconversion of alcohols and aldehydes. Yet, these enzymes have also attracted interest due to their ability to oxidize aldehydes[1]. HLADH was shown to oxidize butanal[2]. This reaction, however, shows no potential for synthetic application unless a very efficient NAD^+ regeneration system is applied (Fig. 16.4-2). The catalytic activity of HLADH for the reduction of the aldehyde is more than 100 times higher than that for aldehyde oxidation (examined for benzaldehyde)[3]. As a result, the initially formed NADH is

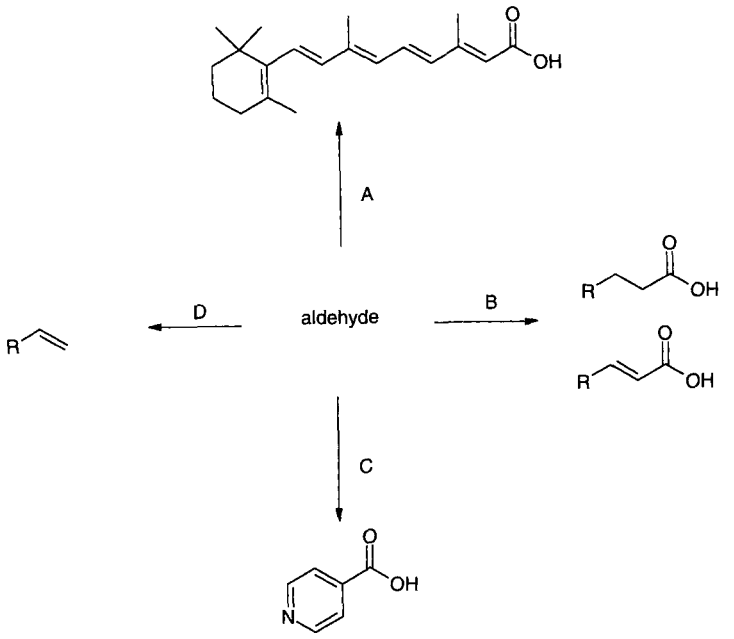


Figure 16.4-1. Selected enzymatic oxidations of aldehydes. A: oxidation of complex natural products such as retinal; B: oxidation of aliphatic and α,β-unsaturated aldehydes; C: oxidation of (hetero)arylic aldehydes; D: oxidative cleavage of the aldehyde-carbon atom yielding terminal alkenes.

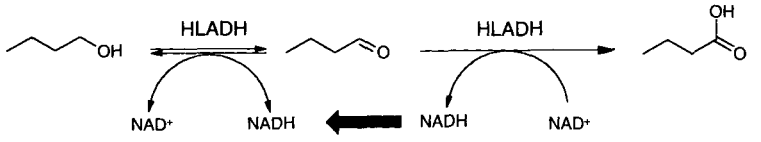


Figure 16.4-2. Oxidation activity for aldehydes exhibited by horse liver alcohol dehydrogenase (HLADH). Only minor amounts of acid are produced because of the higher HLADH activity for aldehyde reduction.

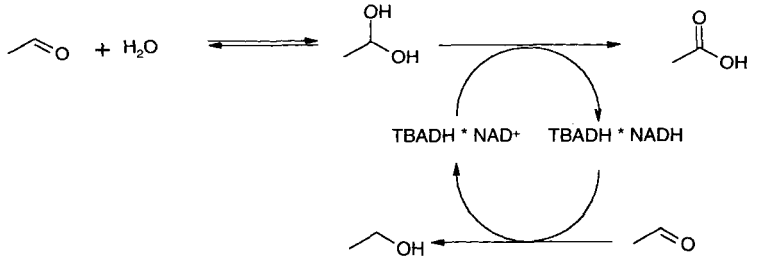


Figure 16.4-3. Aldehyde dismutase acitivity of *Thermoanaerobium brockii* alcohol dehydrogenase (TBADH). A high affinity of the TBADH-NAD$^+$ complex for hydrated acetaldehyde is proposed, explaining the stochiometric acetaldehyde dismutation.

used for aldehyde reduction, yielding a dynamic equilibrium between alcohol and aldehyde.

TBADH also exhibits the so-called aldehyde dismutase activity[4]. In contrast to HLADH, stochiometric dismutation of acetaldehyde into one equivalent of ethanol and acetic acid has been reported. A *gem*-diol mechanism was proposed for this reaction (Fig. 16.4-3).

16.4.3
Aldehyde Dehydrogenases

Several aldehyde dehydrogenases have been reported for biocatalytic applications.

Recently, aldehyde dehydrogenase (E.C. 1.2.1.5) from yeast was applied to oxidize (*Z,Z*)-nona-2,4-dienal[5]. Recycling of NAD^+ was achieved *in situ* by addition of an alcohol dehydrogenase, reducing (*Z,Z*)-nona-2,4-dienal to the corresponding alcohol. Since both reactions are stochiometrically linked via NAD, this corresponds to an overall dismutation of the aldehyde (Fig. 16.4-4). This concept was extended to industrially relevant metabolites of linoleic acid (detergents and polymer building-blocks) (Fig. 16.4-5). No isomerization of the double bonds and yields up to 90% were reported[5].

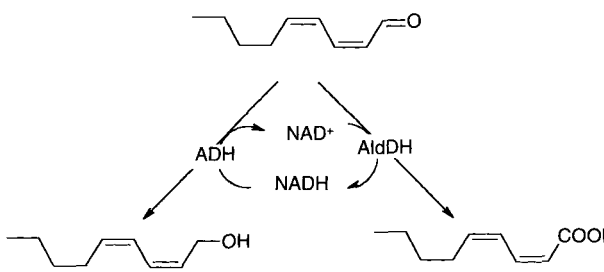


Figure 16.4-4. Enzymatic transformation of (*Z,Z*)-nona-2,4-dienal to the corresponding alcohol and acid catalyzed by an alcohol and an aldehyde dehydrogenase from yeast.

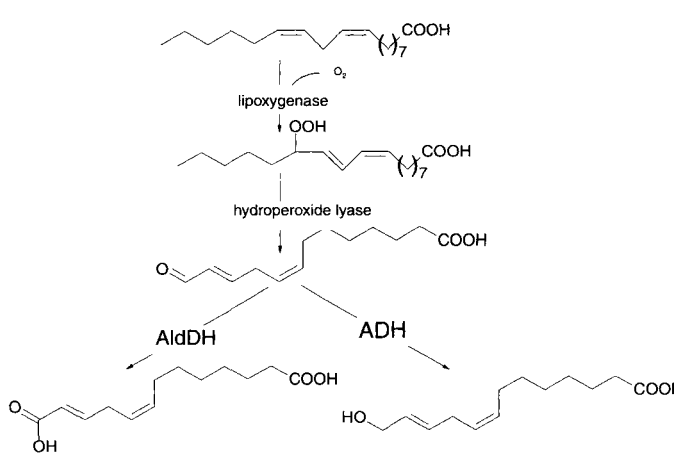
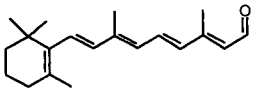
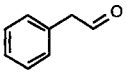


Figure 16.4-5. Enzymatic cleavage of linoleic acid to ω-hydroxy and dicarboxylic acids.

Table 16.4-1. Kinetic constants of bovine kidney aldehyde dehydrogenase for different substrates[1].

Substrate	V_{max} [%][a]	K_M [μM]
	100	9.1
	758	1
	855	1.5
	1960	30
	1683	33.9
	3026	8.2

a The V_{max} values are relative to retinal as substrate.

1 P. V. P. Bhat, L., Wang, X. L., *Biochem. Cell Biol.* **1996**, *74*, 695–700.

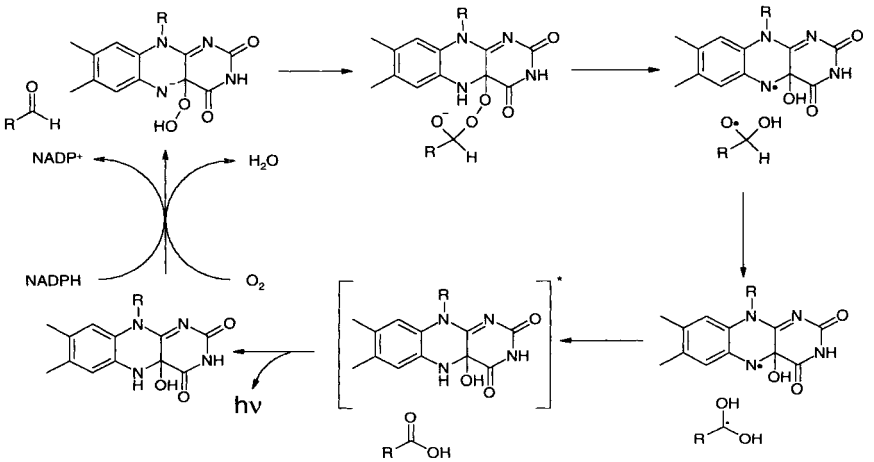
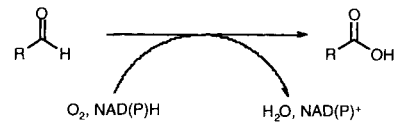
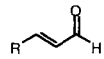
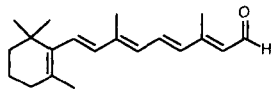
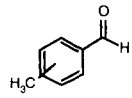
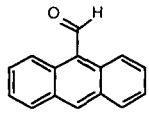
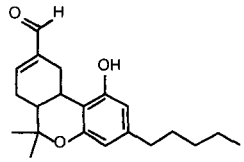


Figure 16.4-6. Mechanism proposed for light emission in the course of the luciferase reaction.

Another NAD^+-dependent aldehyde dehydrogenase (from bovine kidney) was characterized with respect to its activity toward retinal and other aldehydes (Table 16.4-1)[6].

Table 16.4-2. Oxidation of aldehydes to corresponding carboxylic acids catalyzed by P450 monooxygenases.



Substrate	Reference
Aliphatic aldehydes	[2, 3]
	[3]
	[4]
	[5]
	[6]
	[6]
Losartan	[7]

2 Y. Terelius, C. Norsten-Höög, T. Cronholm, M. Ingelman-Sundberg, *Biochem. Biophys. Res. Commun.* **1991**, *179*, 689–694.
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5 K. Watanabe, T. Matsunaga, I. Yamamoto, H. Yashimura, *Drug. Metab. Dispos.* **1995**, *23*, 261–265.
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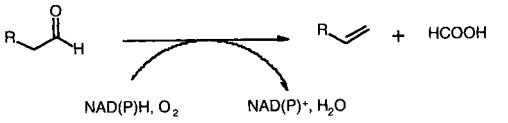
7 R. A. Stearns, P. K. Chakravarty, R. Chen, S.-H. L. Chiu, *Drug. Metab. Dispos.* **1995**, *23*, 207–215.

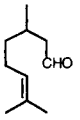
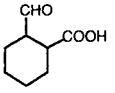
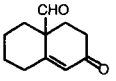
16.4.4 Monooxygenases

16.4.4.1 Luciferase (E.C. 1.14.14.3)

Probably the most prominent oxidation reaction of aldehydes is the well-known luciferase reaction. The flavin-dependent luciferase is present in a number of marine and terrestrial species[7, 9]. Light of about 490 nm (blue-green) is emitted as a by-

Table 16.4-3. Oxidations and subsequent decarboxylations of aldehydes catalyzed by P450 monooxygenases.



Substrate	Reference
	[8]
	[8]
	[9]
	[10]

8 E. S. Roberts, A. D. N. Vaz, M. J. Coon, *Proc. Natl. Acad. Sci USA* **1991**, *88*, 8963–8966.
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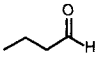
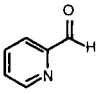
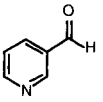
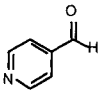
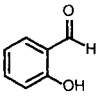
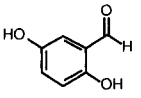
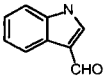
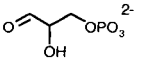
product of the oxidation of aliphatic aldehydes. Excited flavin species are discussed as emitters (Fig. 16.4-6)[9, 10].

16.4.4.2
Cytochrome $P450_{BM-3}$

The oxidation of an aldehyde to the corresponding carboxylic acid with P450 systems is reported for various substrates (Table 16.4-2). In some cases oxidative decarboxylation is observed yielding formic acid and an olefin, one carbon atom shorter than the substrate (Table 16.4-3).

Several ω-oxo fatty acids are transformed to the corresponding α,ω -dicarboxylic acids, whereas ω-formylesters of fatty acids are decarboxylated to the ω-hydroxy fatty acids and carbon dioxide[11]. For several ω-oxo fatty acids turnover frequencies (measured as O_2 consumption) between 1.8 to 25 s^{-1} were found. Many P_{450} systems are multi-component enzymes with small protein cofactors such as putidaredoxin performing the electron mediation between NAD(P)H and the active site of the enzyme. Vilker and coworkers recently were able to show that NADPH can be omitted from the catalytic cycle by direct electrochemical reduction of putidar-

Table 16.4-4. Kinetic constants of xanthine oxidase[11].

Substrate	K_M [mM]	V_{max} [s^{-1}]
	161.5	22.2
	130	100
	430	23.3
	142	2.4
	0.36	3.4
	0.046	2.7
	1.7	4.2
	1.03	7.7
	0.068	15.7
	0.085	1.8
	1	1
	2	0.1

11 F. F. Morpeth, *Biochim. Biophys. Acta* **1983**, *744*, 328–334.

edoxin[12–14], thus oxidizing styrene or camphor. Other approaches utilize Co sepulchrate as reducing agent, which can be regenerated either chemically (via Zn)[15] or electrochemically[16, 17].

16.4.5
Oxidases

16.4.5.1
Xanthine Oxidase (E.C. 1.1.3.22)

Xanthine oxidase was examined for its catalytic applicability for the oxidation of aldehydes as early as 1967[18]. In addition to O_2, xanthine oxidase was reported to accept e.g. methylene blue, PMS or ferricyanide[19] as electron acceptors. Table 16.4-4 gives kinetic data for some substrates[20].

16.4.6
Oxidations with Intact Microbial Cells[21]

Burkholderia cepacia was reported to transform aromatic aldehydes into the corresponding acids. Vanillin, *para*-hydroxybenzaldehyde, and syringaldehyde were converted to corresponding acids with high yields of 94%, 92%, and 72%, respectively (Fig. 16.4-7)[22].

The acid produced is not further metabolized as long as the aldehyde still is accessible to the cells. The enzyme responsible for aldehyde oxidation in *Burkholderia cepacia* was not further characterized. However, the gene of an NAD-dependent vanillin dehydrogenase of *Pseudomonas* sp. strain HR199 was cloned and characterized[23]. Recombinant *E. coli* containing this vanillin dehydrogenase transformed vanillin to vanillate at a clearly higher rate than *Burkholderia cepacia*.

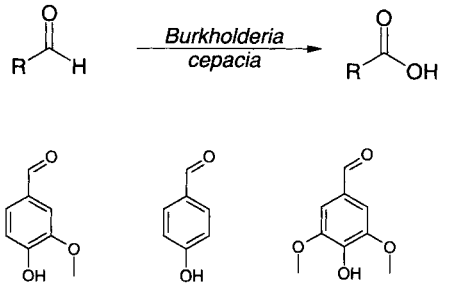


Figure 16.4-7. Oxidation of aromatic aldehydes by *Barkholderia cepacia* TM1.

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23 H. Priefert, J. Rabenhorst, A. Steinbüchel, *J. Bacteriol.* **1997**, *179*, 2595–2607.

16.5
Baeyer-Villiger Oxidations

Sabine Flitsch and Gideon Grogan

16.5.1
Introduction

The enzymatic Baeyer-Villiger oxidation continues to receive attention from synthetic organic chemists as it offers advantages of regio- and enantioselectivity still rarely exhibited by reagents such as *meta*-chloroperbenzoic acid (*m*-CPBA). Some recent advances have resulted in abiotic catalytic reagents capable of inducing modest enantioselectivity in the Baeyer-Villiger reaction [1–3], but these reactions are outside the scope of this section. The most encouraging examples of enantioselective Baeyer-Villiger reactions are still those catalyzed by microorganisms and enzymes and the extensive research in this area over the last decade has been covered in a number of recent reviews [4–7].

16.5.1.1
Steroidal Substrates

It had been known for many years that Baeyer-Villiger-type processes occur during the catabolic transformations of natural compounds. In 1953, it was described that the C17 side chain of steroids can be cleaved by several microorganisms including

Fusarium, *Penicillium*, *Cylindrocarpon*, *Aspergillus* and *Gliocladium* species[8–10]. One example reported was the conversion of progesterone into $\Delta^{1,4}$-androstadien-3,17-dione in 84% yield as illustrated in Fig. 16.5-1 [8].

Since these reports, many others describing the microbiological Baeyer-Villiger oxidation of various steroids have been published[11–14]. Interestingly, it has been shown that depending on the microbial strain used, further oxidation may occur leading to incorporation of an oxygen atom into the D-ring, thus affording the corresponding lactone. In general, these oxidations are restricted to this ring. This selectivity may be due to the fact that the A-ring bears an α, β-unsaturated ketone moiety, which appears to display a different reactivity compared with the other carbonyl functions [15]. Introduction of a Δ^1 double bond also often occurs during these processes. Other examples involving oxidation of the A ring have been described with a *Glomerella fusaroides* strain[16] and with *Gymnoascus reesii*[17]. Thus, eburicoic acid affords a 30% yield of A-secoacid whereas the steroidal alkaloid tomatidine leads to the corresponding ketone as the major product, but a smaller amount of A-seco acid is also obtained. This could well be due to hydrolysis of the lactone which would be formed from Baeyer-Villiger oxidation of the parent ketone Fig. 16.5-2.

The mechanism of these reactions has been studied by several groups. Fonken and coworkers[18] first showed using 21-^{14}C labelled progesterone, that the testosterone acetate formed during degradation of progesterone by *Cladosporium resinae* is not an artefact but is indeed an intermediate in the degradation pathway. Further work by Prairie and Talalay[19] using the strain *Penicillium liliacinum* established the involvement of two enzymes, a Δ^1-dehydrogenase and an NADPH-dependent oxygenase. They also showed that $^{18}O_2$ molecular oxygen is incorporated as the ring oxygen atom of testololactone. Rahim and Sih[20] succeeded in showing that an oxygenase (requiring the presence of oxygen) as well as an esterase were involved in the degradation of the progesterone side-chain. In other studies using the 17α-labelled substrate, Singh and Rahkit[21] showed that retention of the deuterium label at the C17 position occurs and that the molecular oxygen is incorporated into the product (Fig. 16.5-3). More recently, a gene from *Rhodococcus rhodochrous* has been cloned and expressed[22], which encodes for a steroid monooxygenase that inserts an atom of oxygen between the C17 and C20 carbons of progesterone, forming testosterone acetate.

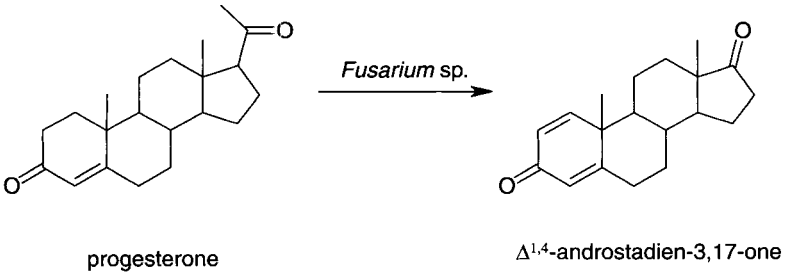


Figure 16.5-1. Biotransformation of progesterone using *Fusarium* spp.

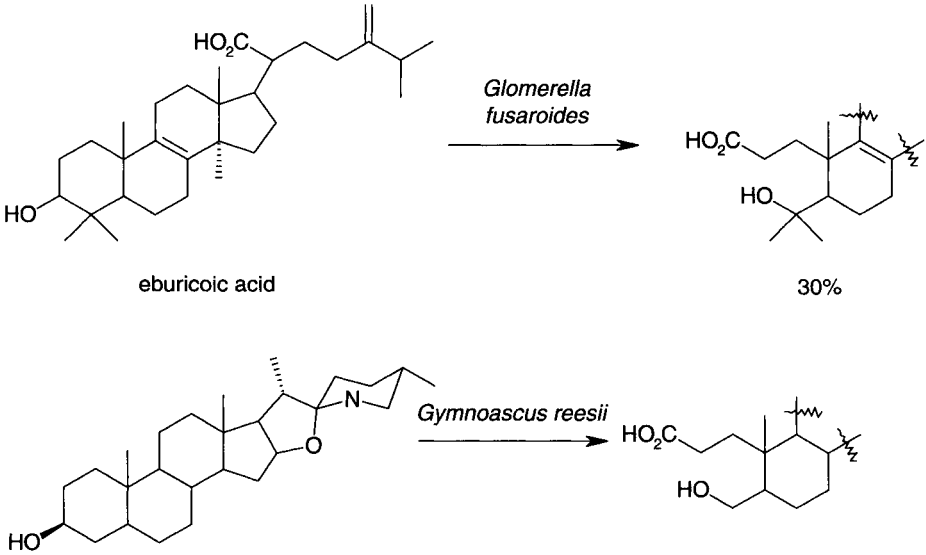


Figure 16.5-2. A-ring cleavage by *Glomerella fusaroides* and *Gymnoascus reesii*.

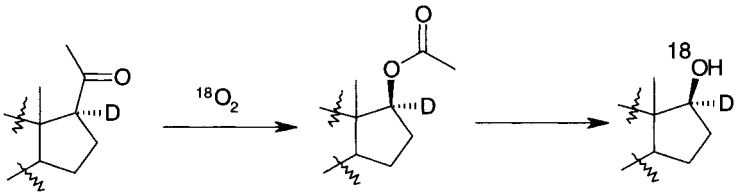


Figure 16.5-3. Retention of the deuterium label and oxygen incorporation during the side-chain degradation of progesterone.

All these results led to the conclusion that a process similar to the Baeyer-Villiger oxidation must occur during these degradations. The general scheme for the formation of testololactone from progesterone can thus be described, as shown in Fig. 16.5-4. It involves four successive steps; first a Baeyer-Villiger oxidation of the steroid sidechain leading to a testosterone acetate, secondly an esterase hydrolysis, thirdly oxidation of the C17 hydroxyl leading to the corresponding 3,17-dione and finally a second Baeyer-Villiger oxidation of this diketone at the D-ring leading to the corresponding δ-lactone. It has been shown in the fungus *Cylindrocarpon radicicola* that one bifunctional enzyme is involved in these transformations, which is able to catalyze oxygenative esterification of 20-ketosteroids as well as oxygenative lactonisation of 17-ketosteroids[23, 24]. It is noteworthy that all the above investigations into steroid substrates for lactonization were conducted on single enantiomers and thus, no reference to the enantioselectivity of the processes had been recorded.

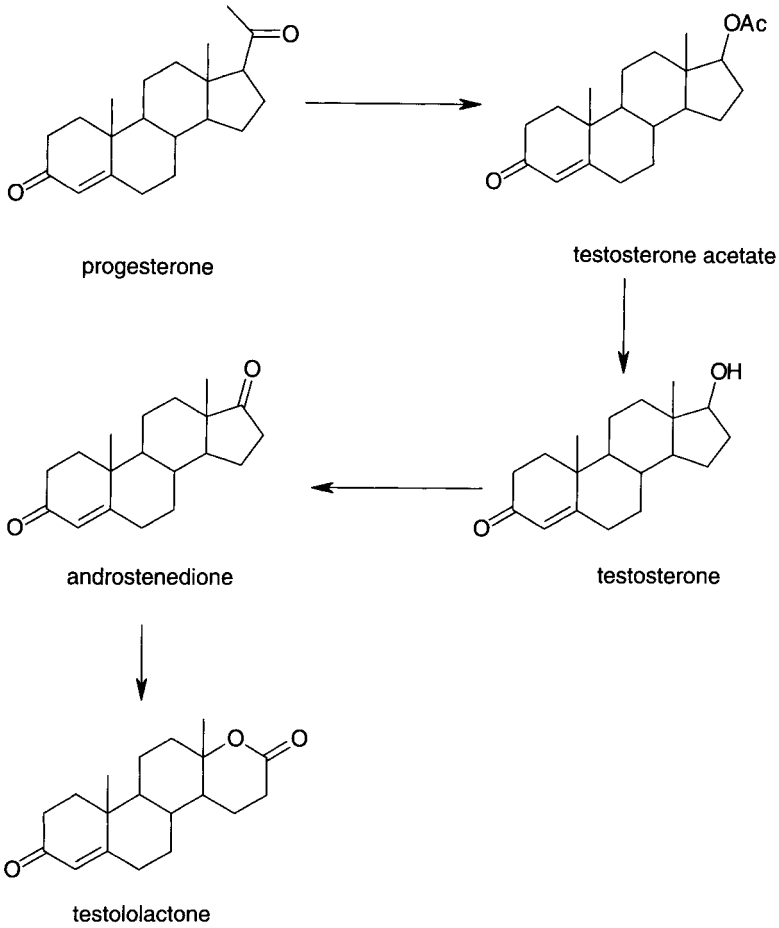


Figure 16.5-4. Mechanism of the biotransformation of progesterone into testololactone.

16.5.1.2
Aliphatic Substrates

Baeyer-Villiger oxidation has also been reported for aliphatic ketones. Several strains able to grow on various aliphatic or alicyclic substrates have been isolated, and it has been shown that their degradation often involves a Baeyer-Villiger oxidation. For example, it has beeen observed that *Pseudomonas multivorans, Pseudomonas aeruginosa, Pseudomonas cepacia* and *Nocardia* sp. are able to grow on tridecan-2-one [25–28].

Forney and Markovetz isolated undecyl acetate directly from growing cultures of *Pseudomonas aeruginosa*. They showed that all early intermediates in the pathway arise biologically and sequentially from their precursors, indicating involvement of a Baeyer-Villiger type oxidation. In a further study they also showed that cell-free

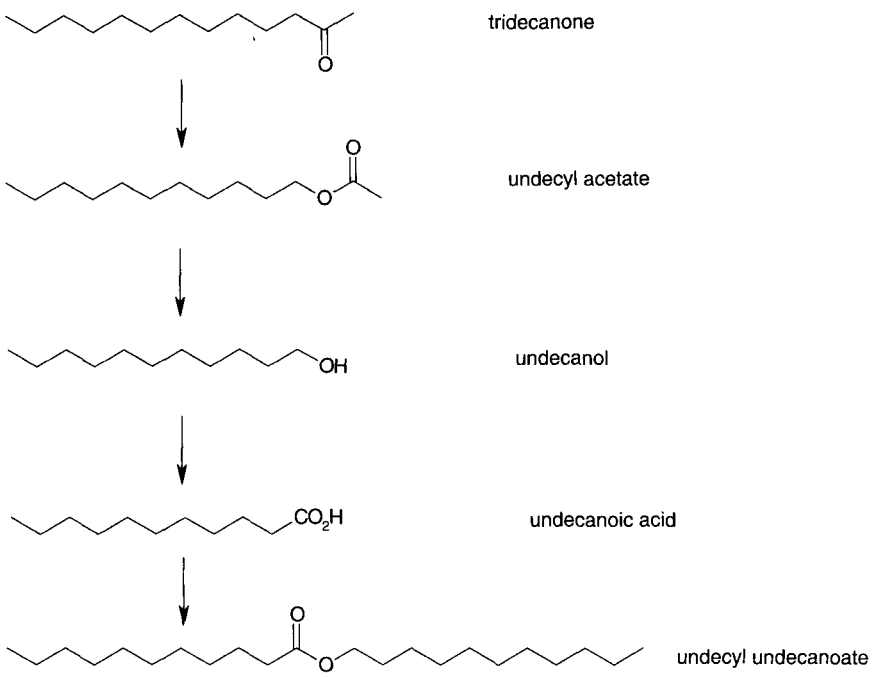


Figure 16.5-5. Degradation of tridecan-2-one with a crude cell-free preparation from a *Pseudomonas aeruginosa* strain.

preparations obtained from methylketone grown *Pseudomonas aeruginosa*, when supplemented with NADH or NADPH in the presence of O_2, carry out a reaction sequence visualized in Fig. 16.5-5.

Using *Pseudomonas cepacia* grown on tridecan-2-one, Markovetz and coworkers [28] later showed that experiments conducted with $^{18}O_2$ led to 84% incorporation of ^{18}O into the C – O – C linkage, rather than into the carbonyl function, indicating the occurrence of a Baeyer-Villiger type process. They also observed that the undecyl esterase involved in the degradation process is able to hydrolyze both aliphatic and aromatic acetate esters. They also reported that this enzyme is strongly inhibited by organophosphates such as tetraethylpyrophosphate (TEPP), as well as by other esterase inhibitors like *p*-chloromercuribenzoate [27].

A similar degradation pathway was described for oxidation of tetradecane and 1-tetradecene with *Penicillium* sp. [29]. Similar mechanisms were proposed for the degradation of other aliphatic substrates such as butan-2-one [28], acetol [30], acetophenone [31] and 1-phenylethanol [32]. Interestingly, cell extracts of *Nocardia* sp. LSU 169 grown on butan-2-one were also shown to be capable of oxidizing tridecan-2-one. Generally, the Baeyer-Villiger reaction was followed by an esterase catalyzed hydrolysis [33].

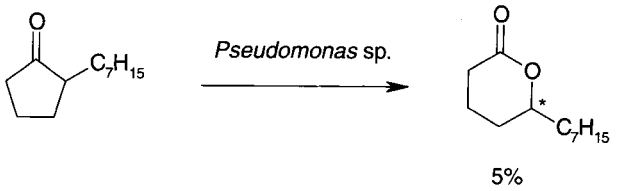


Figure 16.5-6. Degradation of 2-heptyl-cyclopentanone by a *Pseudomonas* sp.

16.5.1.3
Alicyclic Substrates

Baeyer-Villiger oxidation is also a common feature during the catabolic degradation of a variety of other compounds, including monocyclic, bicyclic or polycyclic molecules. For monocyclic compounds, one of the first reports describing formation of a lactone from racemic α-substituted cyclopentanone by various *Pseudomonas* sp. was by Shaw[34]. This could be regarded as the first indication that these reactions were to prove of interest for asymmetric synthesis since the lactone product displayed some optical activity (Fig. 16.5-6).

Further studies showed that other substrates such as cyclopentanol[35], cyclohexane[36–39], cyclohexanol[40–42], cyclohexan-1,2-diol[43–45], cycloheptanone[46] and, more recently, cyclododecane[47] were degraded via analogous pathways. These were studied using bacterial strains including *Pseudomonas* sp. NCIMB 9872[35, 48], *Nocardia globerula* CL1[40], *Acinetobacter* TD 63[43], *Acinetobacter calcoaceticus* NCIMB 9871[39], *Xanthobacter* sp.[38] and *Rhodococcus ruber*[47].

All these degradation pathways were shown to involve a Baeyer-Villiger oxidation of a cycloalkanone that led to formation of the corresponding lactone. Further degradation then occured via hydrolysis of this lactone by a lactone hydrolase which has, in some cases, been isolated. As an example, the reaction sequence for the degradation of cyclopentanol by *Pseudomonas* sp. NCIMB 9872[35] is shown in Fig. 16.5-7.

A pathway for the degradation of (–)-menthol and menthane-3,4-diol by a bacterium classified as a *Rhodococcus* sp. was proposed by Shukla and coworkers. Again, the proposed scheme involves formation of the corresponding lactone by a Baeyer-Villiger process[49]. Interestingly, an identical process has been shown to occur in the degradative pathway of menthol and menthone in peppermint (*Mentha piperita*) rhizomes[50]. *Rhodococcus erythropolis* DCL 14[51] has also been reported to degrade menthone in addition to 1-hydroxy-2-oxo-limonene and dihydrocarvone via an enzymatic Baeyer-Villiger reaction.

Some other monocyclic compounds bearing ketonic side chains have also been shown to undergo degradation processes involving Baeyer-Villiger type oxidation. For example, oxidation of β-ionone by *Lasioplodia theobromae*[52] affords, among other products, the alcohols shown in Fig. 16.5-8. In this case, the loss of two carbons from the sidechain has been attributed to a contribution of Baeyer-Villiger oxidation followed by ester hydrolysis and reduction.

Similar results were described by Nespiak and coworkers[53] in the course of their study of cyclopentyl ketones by *Acremonium roseum* (Fig. 16.5-9). When R = CH_3 or

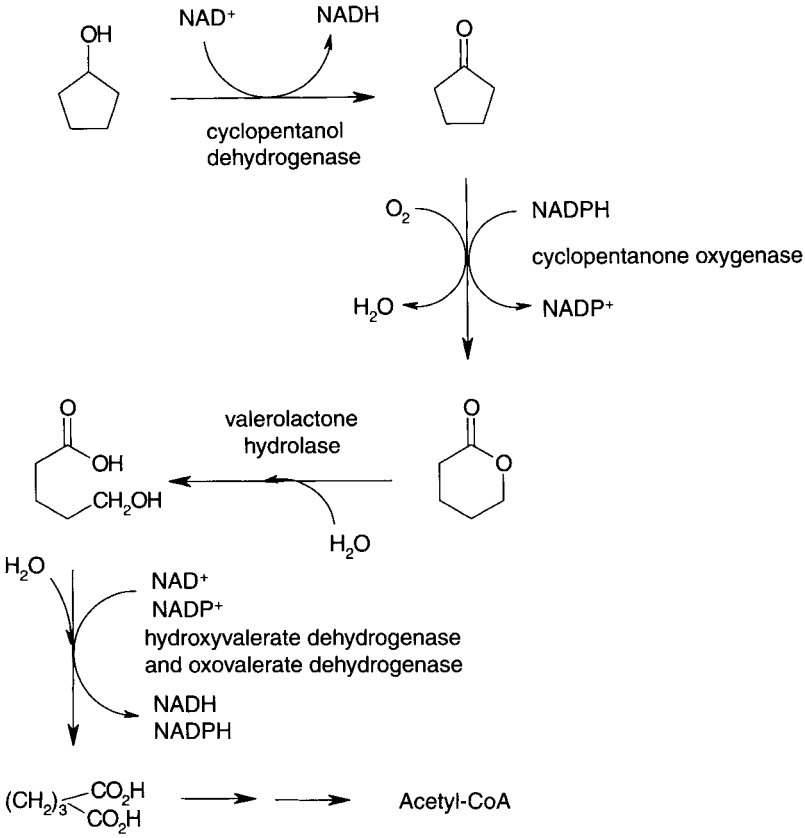


Figure 16.5-7. Reaction sequence for the oxidation of cyclopentanol by *Pseudomonas* sp. NCIMB 9872.

C_2H_5, the alcohol formed via Baeyer-Villiger oxidation and ester hydrolysis was the only product isolated after 2 days. However, higher esters (R = *n*-or *i*-C_3H_7, R = *n*-butyl) have also, if not predominantly, some amount of allylic oxidation product. In this study, it was shown that the (*S*)-enantiomer of the substrate methyl ester was oxidized more rapidly than the (*R*)-isomer and that the reaction proceeded with retention of configuration at the chiral center. Thus, by using short incubation times (2 days) the racemic substrate led to the (*S*)-alcohol, but the optical purity was low (around 20%). Although interesting, this apparent enantioselectivity could also be due eventually to an enantioselective hydrolysis of the intermediate ester or to some other catabolic pathway. However, the butyl ketone led to the (*R*)-alcohol showing 100% optical purity.

An extensive study by Fuganti and coworkers [54–57] showed that the metabolism of 4-(4-hydroxyphenyl)butan-2-one ("raspberry ketone") by the fungus *Beauveria bassiana* unexpectedly yielded tyrosol, through insertion of oxygen via a Baeyer-Villiger reaction and subsequent acetate hydrolysis [54] (Fig. 16.5-10). Only a narrow range of

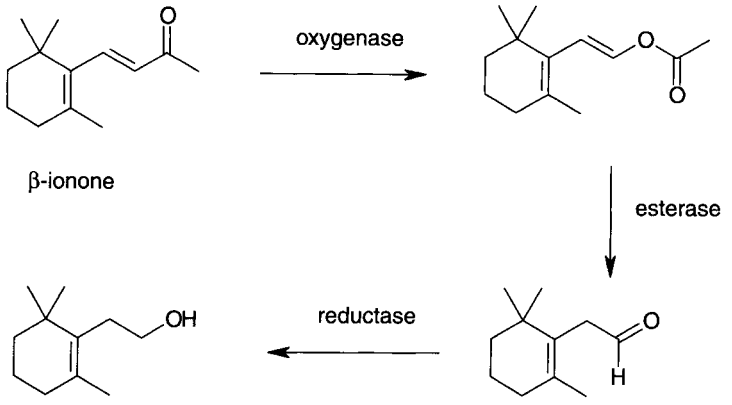


Figure 16.5-8. Biotransformation of β-ionone by *Lasiodiploida theobromae*.

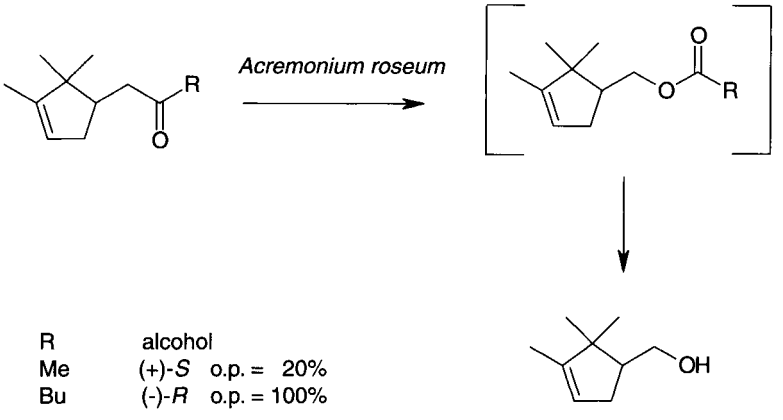


Figure 16.5-9. Transformation of cyclopentyl ketones by *Acremonium roseum*.

substrates was converted in this manner, however[57]. The authors were able to show via deuterium incorporation experiments, that the configuration of the migrating carbon-carbon bond was retained[56], this being a defining characteristic of the peracid-catalyzed Baeyer-Villiger process.

Camphor and its analogs are the most studied bicyclic substrates for the biological Baeyer-Villiger reaction[58–67]. It has been shown by Gunsalus and coworkers that, in the early steps of D-(+)-camphor oxidation by *Pseudomonas putida* C1, both alicyclic rings are cleaved by lactonization reactions: Thus, the conversion of (+)-camphor to 5-keto-1,2-campholide involves three reactions; hydroxylation, oxidation and lactonization. Using a different *Pseudomonas* strain, the non-hydroxylated campholide has been isolated, suggesting that in this case lactonization occurs prior to hydroxylation[58]. It was also shown, by analysis of extracted metabolites, that an analogous, enantiocomplementary pathway existed for the metabolism of L-(−)-camphor. Several further studies have been devoted to clarifying these steps. Interestingly, it has been shown that *P. putida* does not express lactone hydrolases that are active towards

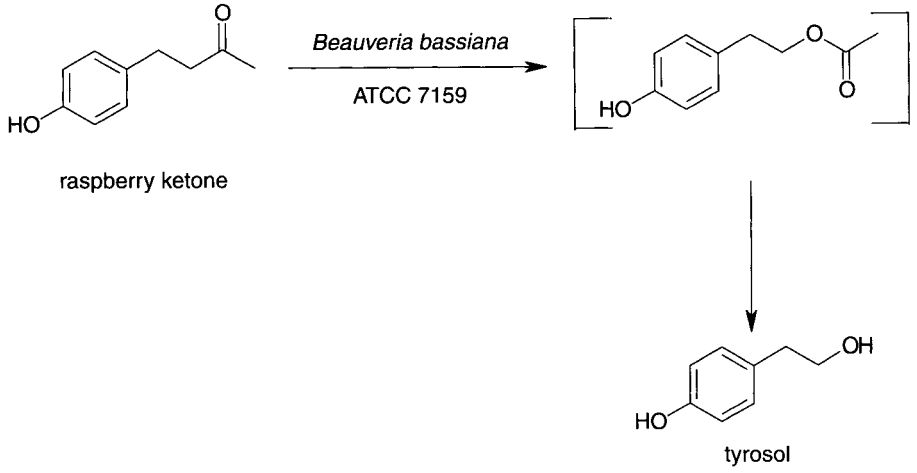


Figure 16.5-10. Biotransformation of raspberry ketone to tyrosol by *Beauveria bassiana* ATCC 7159.

the lactone intermediate. The intermediate bicyclic lactone is unstable under reaction conditions, and spontaneously opens to a cyclopentenone. This is then again oxidized via a Baeyer-Villiger reaction to the corresponding lactone. The degradation of the enantiomers of camphor is shown in Fig. 16.5-11. Three enzymes catalyzing the Baeyer-Villiger reaction, i.e. 2,5-diketocamphane 1,2-monooxygenase [which forms the bicyclic lactone analog of (+)-camphor][64], 3,6-diketocamphane 1,6-monooxygenase [which forms the bicyclic lactone analog of (–)-camphor][67] and 2-oxo-Δ^3–4,5,5-trimethylcyclopentenylacetyl-Co-A monooxygenase (which catalyzes the lactonization of the monocyclic intermediate)[63] from *Pseudomonas putida* ATCC 17453 have been purified to homogeneity and thoroughly characterized.

Enzymatic Baeyer-Villiger oxygenations are not restricted to microbial cells. It has been shown that (+)-camphor, a major constituent of the volatile oil of immature sage (*Salvia officinalis* L.) leaves, is converted into a water soluble metabolite via enzymatic lactonization to 1,2-campholide, followed by conversion into the β-D-glucoside-6-*O*-glucose ester of the corresponding hydroxy acid[68, 69].

The oxidation of racemic fenchone by a *Corynebacterium* sp.[70] (reclassified as *Mycobacterium rhodochrous*), an organism which grows at the expense of either (+)- or (-)- camphor, has also been reported. This was shown to lead, in a 45% yield, to a 90/10 mixture of 1,2 and 2,3-fencholides, as shown in Fig. 16.5-12. This result contrasts with the chemical oxidation of fenchone with peracetic acid, where 2,3-fencholide is the major product in a 40/60 mixture. Accumulation of these lactones is *a priori* surprising as compared with the total degradation of the structurally similar camphor substrate. However this may simply be due to the fact that this lactone, unlike that formed from camphor, is chemically stable in the medium. Of course, one has also to assume that, here again, the strain is devoid of any lactone hydrolase. This bioconversion was the first gram-scale preparative report

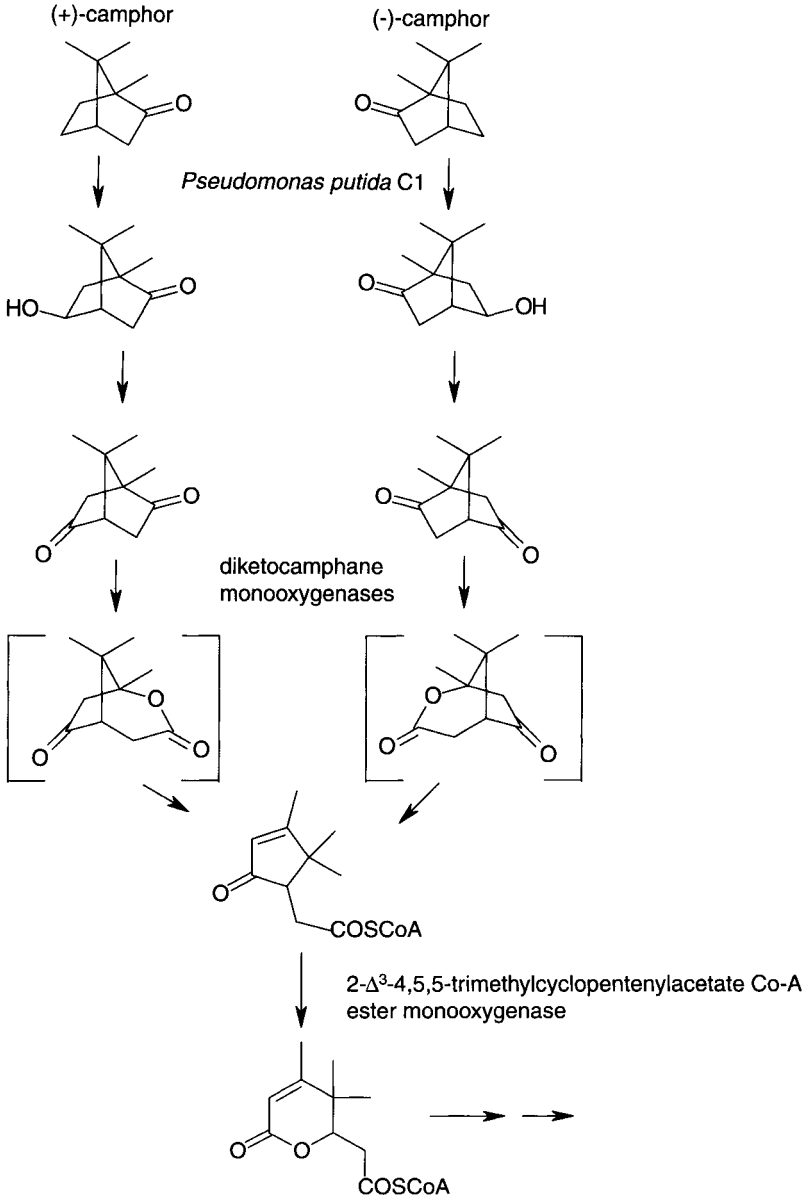


Figure 16.5-11. Metabolism of both enantiomers of camphor by *Pseudomonas putida* C1 (= NCIMB 10007 = ATCC 17453).

of a non-steroidal product, yet no indication of any enantioselectivity for this reaction was presented.

Similarly, it has been shown that 1,8-cineole and 6-oxo-cineole are degraded via the scheme shown in Fig. 16.5-13 [71]. As in the case of camphor, the first step involves a

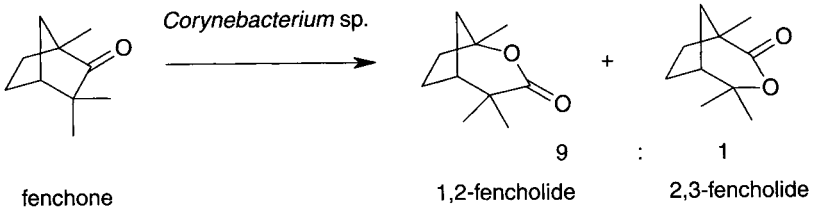


Figure 16.5-12. Oxidation of racemic fenchone to the corresponding fencholides.

hydroxylation, followed by oxidation of the alcohol to form 6-oxocineole. This is then processed via a Baeyer-Villiger reaction leading to a lactone which is spontaneously opened to the hydroxy acid.

16.5.1.4
Polycyclic Molecules

Enzymatic Baeyer-Villiger reactions have also been described in the metabolic processing of larger, polycyclic non-steroidal molecules (Fig. 16.5-14). This is the case for the biosynthesis of aflatoxin B1 where it has been demonstrated that formation of versiconal acetate intermediate from averufin occurs via such a process[72]. Similarly, aflatoxin G1 was shown to be formed from aflatoxin B[73] whereas degradation of the anthraquinone questin to desmethylsulochrin was shown to imply a Baeyer-Villiger process[74].

Furthermore, biological Baeyer-Villiger reactions have been reported in the biosynthesis of polyketides such as DTX-4[75] and the aureolic acid antibiotics such as mithramycin[76, 77]. The oxygenase MtmOIV from *Streptomyces argillaceus* responsible for cleavage of the fourth ring of premithramycin B is unique amongst those responsible for biological Baeyer-Villiger reactions, in that it displays sequence homology not with other "Baeyer-Villiger monooxygenases" (*vide infra*), but with flavin-type hydroxylases encoded in polyketide synthase gene clusters from other *Streptomyces* spp.[76].

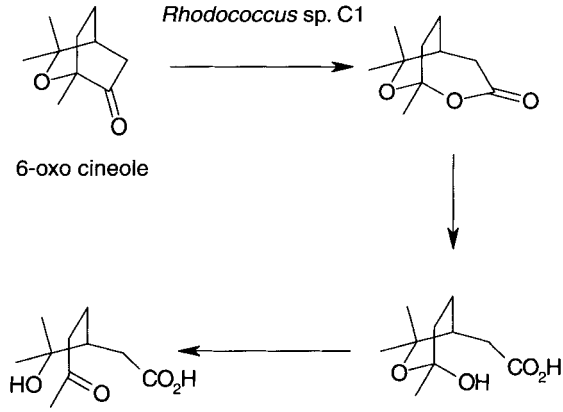


Figure 16.5-13. Degradation of 6-oxo-cineole by a *Rhodococcus* sp.

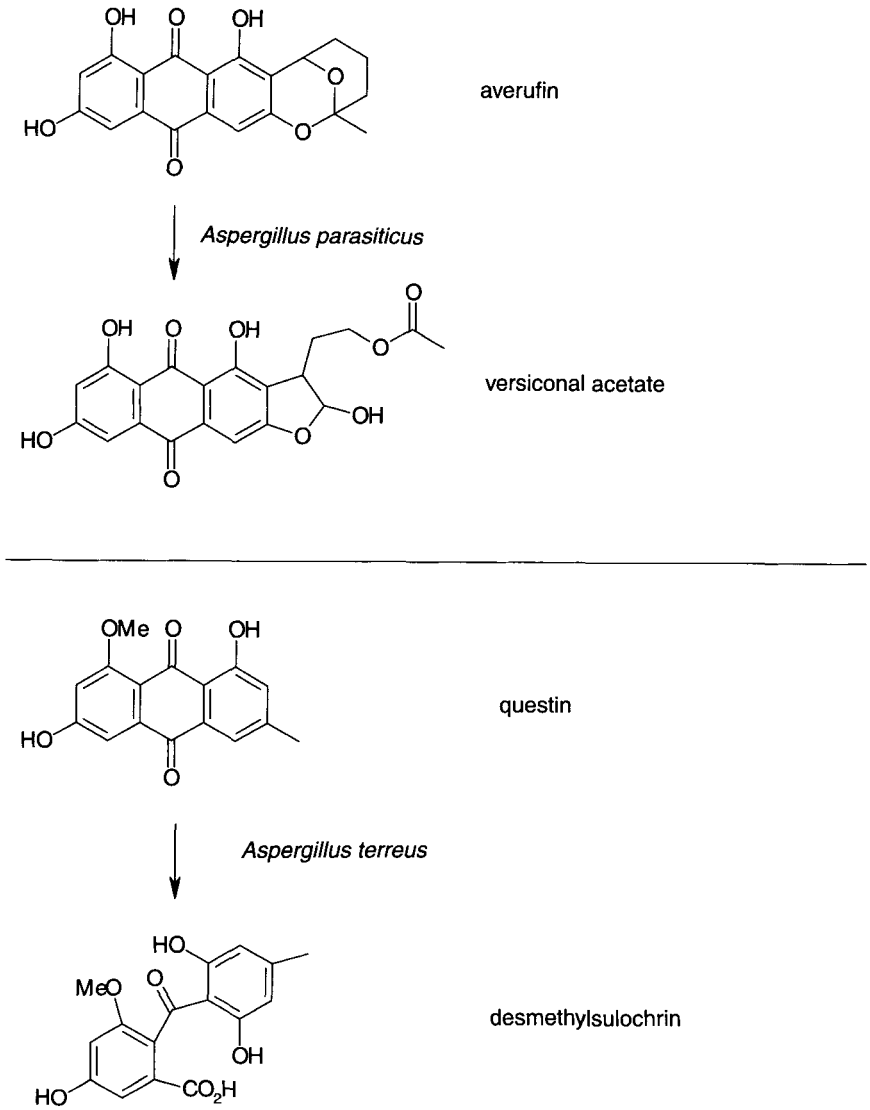
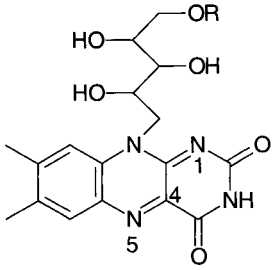


Figure 16.5-14. Involvement of enzymatic Baeyer-Villiger processes in the degradation of non-steroidal polycyclic compounds.

16.5.2
Baeyer-Villiger Monooxygenases

The reactions described above illustrate that there are numerous metabolic routes wherein biological Baeyer-Villiger reactions have been implicated. The synthetic potential of the enzymatic Baeyer-Villiger reaction has dictated that intensive



R = H riboflavin
R = PO^{2-}_3 FMN
R = ADP FAD

Figure 16.5-15. Riboflavin derivatives: the coenzymically active forms of flavoprotein.

research efforts have been devoted to studying the nature of the enzymes that catalyze these reactions.

Enzymes that catalyze the Baeyer-Villiger reaction are a subset of the flavin monooxygenases. In the mechanism of oxidation catalyzed by such enzymes one atom of molecular oxygen is incorporated into the substrate, whereas the other is reduced to H_2O. Two cofactors are required for catalytic activity. The first is a reduced flavin (FAD or FMN) bound non-covalently in the active site. The riboflavin moiety of flavin monooxygenase holoproteins is shown in Fig. 16.5-15; the second is a reduced nicotinamide cofactor (NADPH or NADH), which is required to furnish the enzyme with electrons to reduce the flavin.

Several Baeyer-Villiger monooxygenases (BVMOs) have been purified and in rare cases, the relevant genesm cloned and expressed. Some of these are listed in Table 16.5-1. There appear to be two types of BVMOs. Type 1 are homogeneous; both flavin reduction and substrate oxygenation are carried out on a single polypeptide, these are most usually FAD and NADPH dependent. Type 2 are heterogeneous, a substrate oxygenating subunit appears to require a separate flavin reductase/NADH dehydrogenase in order to generate reduced flavin. Type 2 BVMOs are usually FMN and NADH dependent.

16.5.2.1
Type 1 BVMOs

Cyclopentanone monooxygenase, which catalyzes the conversion of cyclopentanone to valerolactone, has been isolated from *Pseudomonas* sp. NCIMB 9872[46, 78]. This has been shown to be made up of three identical subunits, each using one FAD equivalent, and to be NADPH dependent. Cyclohexanone monooxygenases have been purified from *Acinetobacter calcoaceticus* NCIMB 9871 and *Nocardia globerula* CL1[40] and *Rhodococcus coprophilus*[79]. These enzymes were shown to be single polypeptides and to be FAD and NADPH dependent. Tridecanone monooxygenase from *Pseudomonas cepacia* is a dimer of two identical subunits, however, but is also FAD plus NADPH dependent[80]. A cyclohexanone monooxygenase from *Xanthobacter* sp. is unusual in that it is dependent on FMN, but NADPH as a nicotinamide cofactor[82]. Steroid monooxygenase from *Rhodococcus rhodochrous*[83] and monocyclic monoterpene ketone monooxygenase from *Rhodococcus erythropolis* DCL 14[51]

Table 16.5-1. Characteristics of various Baeyer-Villiger monooxygenases.

Enzyme and source	Number of proteins	Subunit structure	Cofactor specificity	Native molecular mass × 1000 Da	Mole of flavin/ mole of protein	Optimum pH	Reference
Cyclopentanone monooxygenase *Pseudomonas* NCIMB 9872	1	3–4 identical subunits	NADPH	200 (54–58 each)	1 FAD per subunit	7.7	[48]
Cyclohexanone monooxygenase *Acinetobacter calcoaceticus* NCIMB 9871	1	single polypepetide	NADPH	59	1 FAD	9.0	[81]
Cyclohexanone monooxygenase *Nocardia globurela* CL 1	1	single polypeptide	NADPH	53	1 FAD	8.4	[81]
2-Tridecanone monooxygenase *Pseudomonas cepacia*	1	2 identical subunits	NADPH	123 (55 each)	1 FAD	7.8–8.0	[80]
2-Oxo-Δ^3-4,5,5-trimethylcyclopentenyl acetyl Co-A monooxygenase *Pseudomonas putida* ATCC 17453	1	2 identical subunits	NADPH	106	1 FAD	9.0	[63]
2,5-Diketocamphane 1,2-monooxygenase *Pseudomonas putida* ATCC 17453	2	2 identical substrate oxygenating subunits + NADH dehydrogenase	NADH	78 (39 each)	1 FMN per subunit	7.2	[64]
3,6-Diketocamphane 1,6-monooxygenase *Pseudomonas putida* ATCC 17453	2	2 identical substrate oxygenating subunits + NADH dehydrogenase	NADH	72 (36 each)	1 FMN per subunit	–	[67]
Steroid monooxygenase *Cylindrocarpon radicicola* ATCC 11011	1	2 identical subunits	NADPH	115 (56 each)	1 FAD per subunit	7.8	[83]
Cyclohexanone monooxygenase *Xanthobacter* sp.	1	single polypeptide	NADPH	50	FMN	8.8	[82]
Monocyclic monoterpene ketone monooxygenase *Rhodococcus erythropolis* DCL 14	1	single polypeptide	NADPH	60	FAD	9.0	[51]
Cyclohexanone monooxygenase *Rhodococcus coprophilus*	1	single polypeptide	NADPH	58	FAD	–	[79]
Steroid monooxygenase *Rhodococcus rhodochrous*	1	single polypeptide	NADPH	60	FAD	–	[83]

are also Type 1 BVMOs as is the 2-oxo-Δ^3-4,5,5-trimethylcyclopentenyl acetyl Co-A monooxygenase from *Pseudomonas putida* ATCC 17453[63].

Cyclohexanone monooxygenase (CHMO, E.C. 1.14.13.X) is by far the most studied Type 1 BVMO and has been used extensively for as a model for mechanistic studies and as a catalyst in synthesis (*vide infra*). CHMO was purified from *Acinetobacter calcoaceticus* NCIMB 9871 grown on cyclohexanol as the sole carbon source, by Trudgill and coworkers[81]. It was found to be active as a monomer and to contain one non-covalently bound FAD molecule per monomer. The gene was cloned and the protein expressed in *Escherichia coli*[84] and more recently in *Saccharomyces cerivisiae*[85]. Each subunit is a polypeptide of 542 amino acids and, although no definitive structure of a BVMO has yet been published, a potential flavin binding site at the *N*-terminus was identified, in addition to a potential NADP binding site. Analysis of the sequence reveals that the N-terminus of the enzyme bears strong homology with the FAD binding domain of other flavoproteins such as glutathione reductase from *Escherichia coli*.

16.5.2.2
Type 2 BVMOs

The diketocamphane monooxygenases (DKCMOs) from *Pseudomonas putida* ATCC 17453 involved in camphor degradation, are FMN plus NADH dependent and are heterogeneous, consisting of two identical substrate oxidizing polypeptides and an NADH dehydrogenase. The enzymes have been purified, extensively characterized[64, 67] and their N-terminal amino acid sequences determined[86]. These data showed the oxygenating subunits of the DKCMOs to have homology with the NADH plus FMN dependent luciferase of *Vibrio harveyi*[87], an enzyme which catalyzes the Baeyer-Villiger oxidation of dodecanal to dodecanoic acid with the release of a photon of light. The application of the DKCMOs enzymes to synthesis has also been investigated (*vide infra*) and, whilst the genes encoding these proteins have not been identified, preliminary X-ray crystallographic data on 3,6-diketocamphane-1,6 monooxygenase has been reported[88].

16.5.2.3
Mechanism of the Enzymatic Baeyer-Villiger Reaction

The mechanism of the enzymatic Baeyer-Villiger oxidation, with reference to CHMO, has been studied by the group of Walsh[73, 89] who proposed the scheme shown in the top cycle in Fig. 16.5-16. The tricyclic isoalloxazine ring is the center of catalysis. Initially, the exogenous reductant NAD(P)H acts as the electron donor to afford the reduced flavin. This can be readily reoxidized by both one-electron or two electron processes in the presence of O_2 to yield a 4-*a*-hydroperoxyflavin. This intermediate undergoes an O-O bond fission upon nucleophilic attack on an electrophilic ketone substrate, a mechanism similar to the chemical Baeyer-Villiger oxidation of ketones by peracids. This initially affords the 4-*a*-hydroxyflavin which, by loss of H_2O regenerates the starting FAD for a subsequent catalytic cycle.

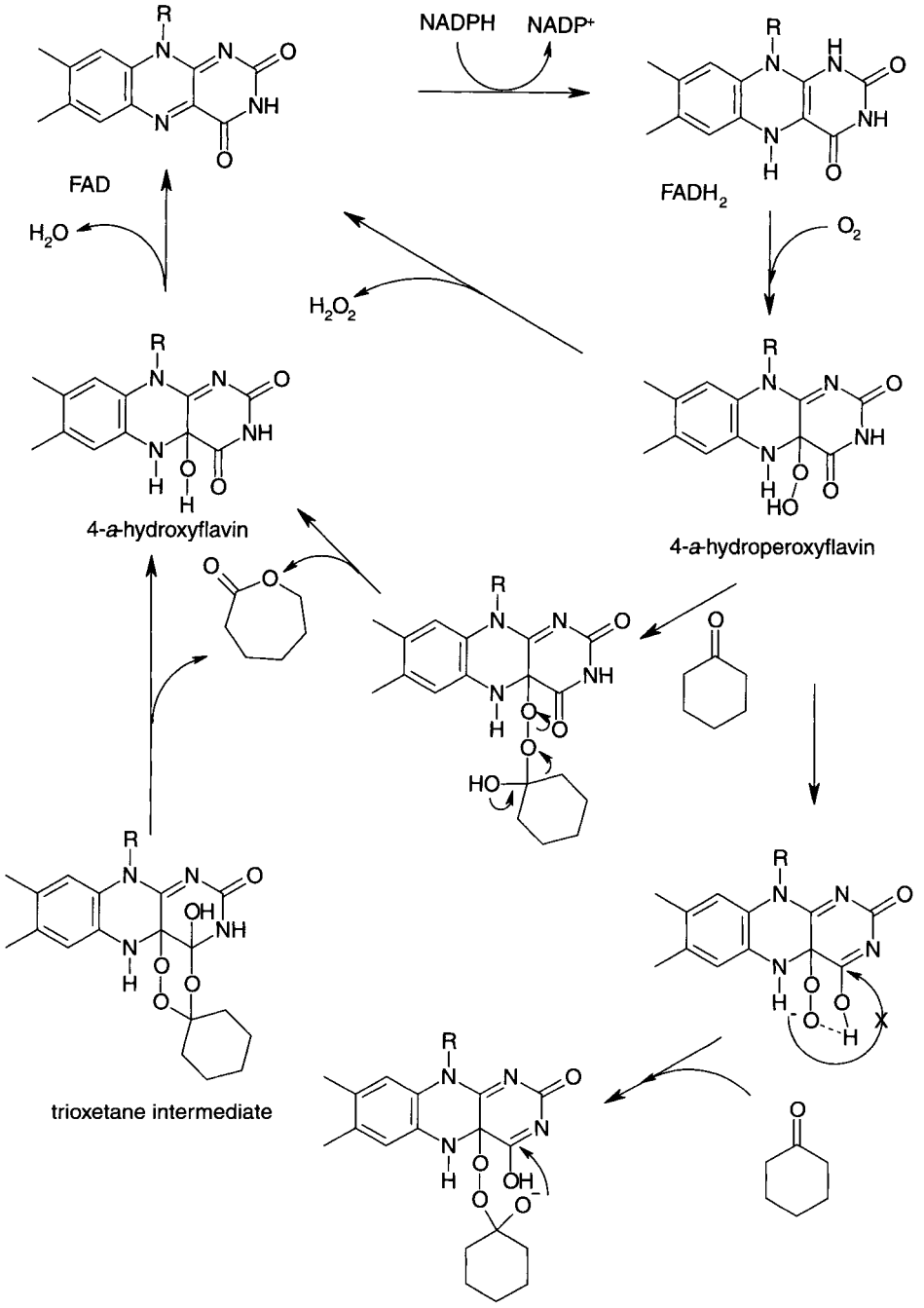


Figure 16.5-16. Proposed mechanisms for the enzymatic Baeyer-Villiger oxidation of cyclohexanone.

However, the FAD-4-*a*-OOH can also break down directly via liberation of H_2O_2.

A variation on this model has recently been proposed by Kelly et al.[7] who suggested that the hydroxy group of the Criegee intermediate could not be immobilized in such a mechanism, and that unreasonable steric constraints would be imposed for many of the substrates transformed reported for these enzymes. A new tautomer of the the flavin hydroperoxide was proposed as part of an alternative scheme (lower cycle, Fig. 16.5-16) in which an intermediate trioxane decomposes to yield the lactone and flavin hydrate.

In addition to ketone substrates, the 4-*a*-hydroperoxyflavin can also react by nucleophilic attack on other molecules. Thus, boronic acid substrates were transformed into the corresponding alcohols via the intermediate borate esters as hydrolytically labile initial enzyme products[90, 91].

However, the 4-hydroperoxyflavin, acting in these cases as an electrophile, is also able to oxidize other nucleophilic substrates and in particular heteroatoms such as sulfur[91], selenium[91, 92], nitrogen[73] and phosphorous[91]. Indeed, CHMO oxygen-

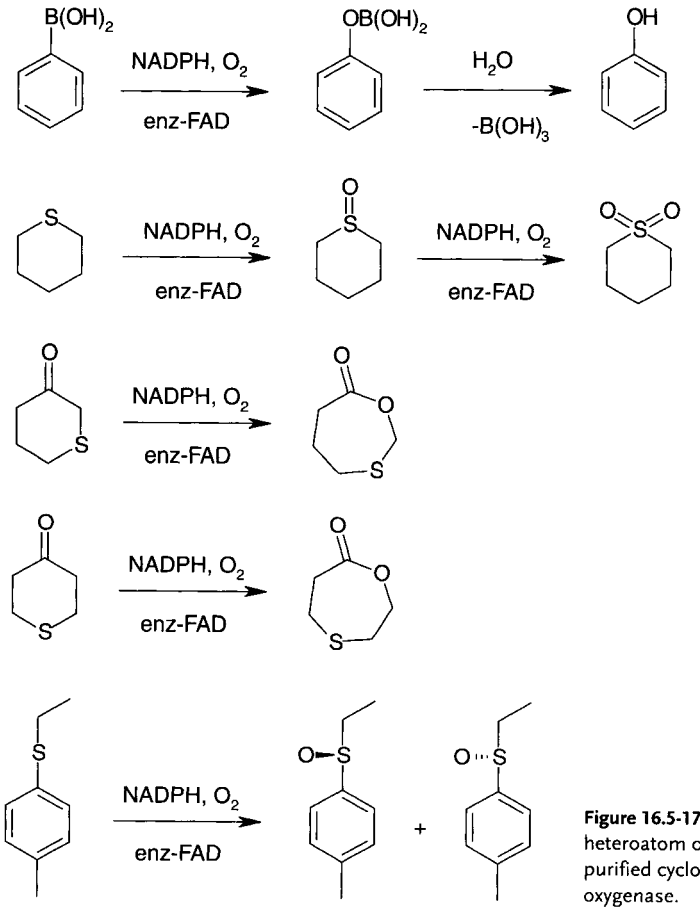


Figure 16.5-17. Studies on heteroatom oxidation using purified cyclohexanone monooxygenase.

ates trimethyl phosphite to trimethyl phosphate, sulfides to sulfoxides (one equivalent) or sulfones (two equivalents). If 3- or 4-thiocyclohexanones were used as substrates, these were converted exclusively into the lactone products, showing that Baeyer-Villiger oxidation is preferred in these cases (Fig. 16.5-17). The synthetic applications of heteroatom, notably sulfur, oxidation by BVMOs have been thoroughly explored and and reviewed[93].

These results illustrate that reactions performed by BVMOs are similar to those of peroxide containing reagents (hydrogen peroxide, alkyl hydroperoxides or peracids), which are able to deliver either a formally nucleophilic or a formally electrophilic oxygen atom to a substrate. Indeed, whereas Baeyer-Villiger oxidation or boronic acid oxygenation involve initial attack of a nucleophilic oxygen, the sulfide, selenide or phosphite ester oxygenations require the transfer of an electrophilic oxygen to a nucleophilic electron pair of the substrate. Interestingly, no epoxidation of olefinic double bonds by BVMOs have been reported however[73].

The substrate selectivity of CHMO was first explored by Trudgill and coworkers[70, 81], who demonstrated that the enzyme processes C4-C8 cyclic ketones. The migratory aptitude of the enzymatic oxygen insertion process was probed initially with two types of substrates. First, in an attempt to explore the stereochemical mode of these reactions, Schwab *et al.* studied the Baeyer-Villiger oxidation of (2*R*)-deuterated cyclohexanone. Detailed NMR multinuclear spectroscopic studies led to the conclusion that CHMO catalyzes the conversion of cyclohexanone to ε-caprolactone with complete retention of configuration at the migrating carbon center[94], a result identical to the chemical route (Fig. 16.5-18). To eliminate the possibility of an enolization and/or rearrangement route, 2,2,6,6-tetradeuterocyclohexanone was also incubated with the enzyme. The fact that no loss of deuterium was observed by GC again militates in favor of a mechanism similar to that proposed for chemical Baeyer-Villiger oxidation.

In an elegant further study[95], these authors confirmed their preliminary proposal of the (*R*)- absolute configuration of the starting 2-deuterocyclohexanone as well as the occurrence of a total retention of configuration of the CHMO catalyzed Baeyer-

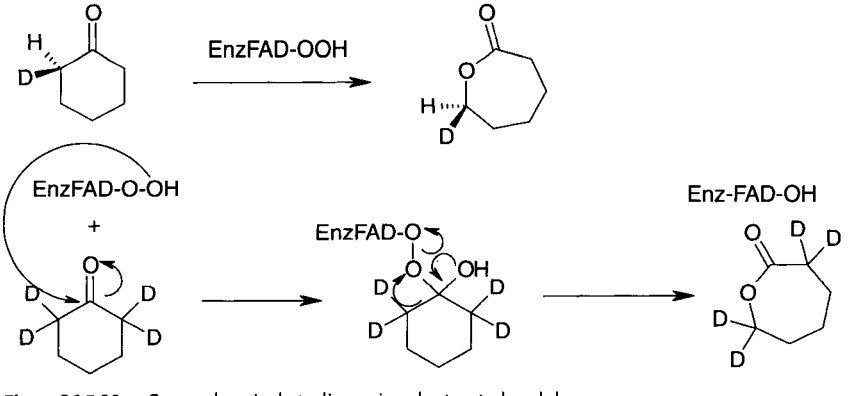


Figure 16.5-18. Stereochemical studies using deuterated cyclohexanone.

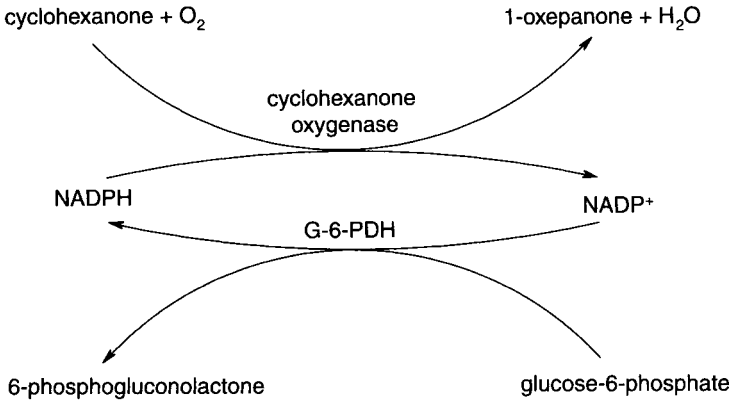


Figure 16.5-19. NADPH recycling in the course of CHMO catalyzed oxidation.

Villiger reaction. Interestingly, they described for the first time that the efficient conversion of ketone into lactone could be brought about in the presence of a catalytic amount of NADPH, with cofactor recycling accomplished by the glucose-6-phosphate dehydrogenase as shown in Fig. 16.5-19.

In order to test enzyme regio- and enantioselectivity rigorously, Schwab and co-workers also studied the asymmetric substrate 2-methylcyclohexanone. A "virtual racemate" made up of equivalent quantities of (2*R*)-2-[methyl-$^2H_3$]methylcyclohexanone and of (2*S*)-[methyl-^{13}C]methylcyclohexanone (each one prepared by different methods) was studied, using a multinuclear NMR technique. The conclusions from this experiment were two fold. First, they confirmed that 6-methyl-ε-caprolactone is the only reaction product, thus indicating a total regioselectivity of oxygen insertion into the "more substituted" carbon-carbon bond. Second, these results showed for the first time a two-fold rate difference between transformation of the two enantiomers of cyclohexanone. This was an interesting result that suggested that CHMO could show far greater discrimination toward enantiomers of a substrate that bore a far bulkier C2 substituent. Finally, the measurement of the reaction kinetics for each one of the substrate enantiomers showed that, after about 50% reaction, there is a progressive decrease in both the degree of enantioselectivity as well as the absolute rate of lactonization of the two substrate enantiomers. However, the reasons for these diminishing rates of reaction were not clear.

Further work exploring the migratory aptitude of different substituents has been described[91]. Phenylacetone is converted into benzyl acetate, showing exclusive benzyl migration in accordance with the chemical reaction achieved with trifluoroacetic acid. Different results were observed with phenacetaldehyde, where an inverted preference is seen as compared with the peracidic reactions.

Using purified CHMO from *A. calcoaceticus* NCIMB 9871, Taschner and coworkers[96, 97] showed that several prochiral substrates including some 4-substituted cyclohexanones were efficiently converted into their corresponding lactones, each of them showing very high enantiomeric purities (Fig. 16.5-20). Thus, CHMO prove to

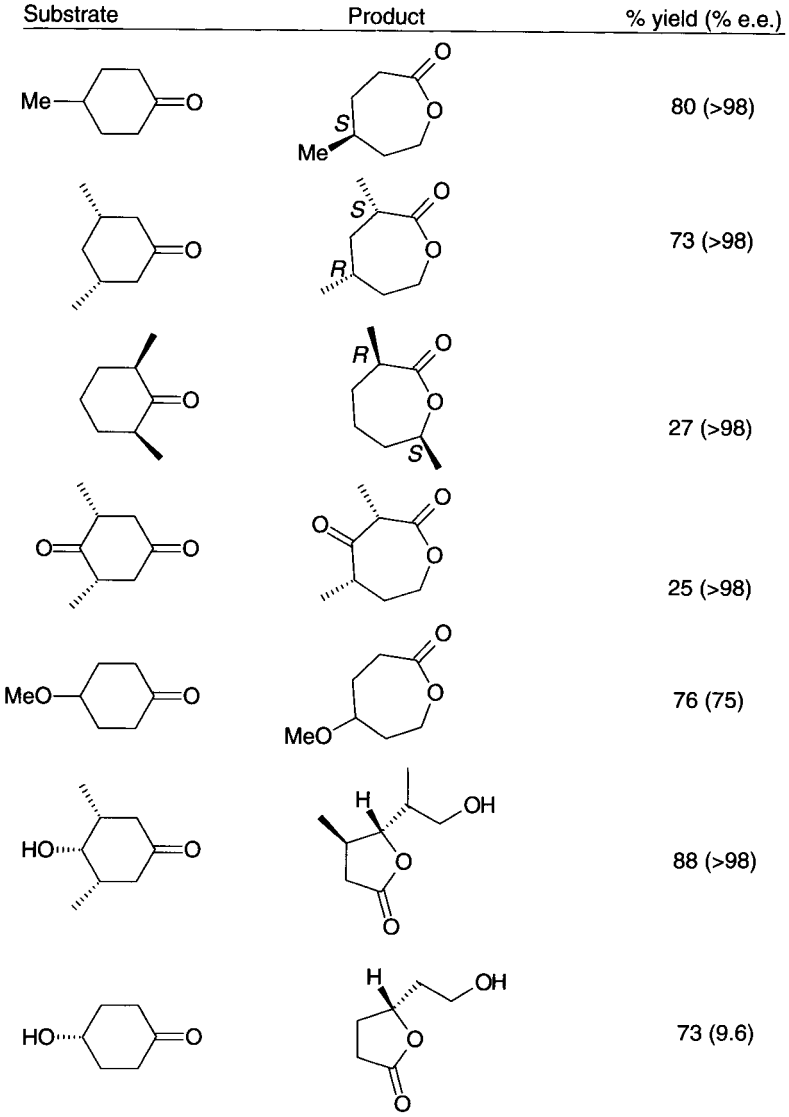


Figure 16.5-20. CHMO catalyzed oxidation of various prochiral substrates.

be extremely effective at discriminating between the two sides of the carbonyl function of such prochiral substrates. However, the presence of an alcohol or methyl ether function at position 4 leads unexpectedly to products of lower ee values.

16.5.3
Synthetic Applications

With the exception of steroid type substrates, the results described up to now have dealt with small-scale analytical studies. However, in view of the potential of BVMOs for regio- and even enantioselective transformations of various substrates, studies into the scale-up of these transformations began in earnest soon after these earlier investigations, followed by considerations of their application in chiral organic synthesis.

In this context, Abril et al.[98] examined a variety of readily available ketones in order to determine the substrate selectivity, regioselectivity and enantioselectivity of CHMO immobilized in a polyacryalamide gel. They also used the NADPH recycling system previously desribed by Schwab for *in situ* regeneration of this cofactor. These experiments showed that 2-norbornanone, L- and D-fenchone, (+)-camphor and (+)-dihydrocarvone are processed by CHMO. In a typical experiment, 10.2 g of racemic 2-oxabicyclo[3.2.1]octan-3-one were obtained from 11.4 g of 2-norbornanone, using 1.7 g of NADP cofactor. The authors concluded that the enzyme did not display a useful degree of enantioselectivity, therefore offering no major advantages over chemical oxidation.

One major drawback of employing CHMO as a catalyst is the necessity to regenerate the expensive nicotinamide cofactor NADPH. One strategy for circumventing this problem is use of whole-cell preparations of microorganisms for Baeyer-Villiger oxidations. One early example of this technique involved the oxidation of 2,2,5,5-tetramethyl-1,4-cyclohexanedione to the optically pure (*S*)-ketol by *Curvularia lunata* described by Azerad and coworkers[99]. They showed that during the fungal reaction of the dione, as shown in Fig. 16.5-21, the already formed (*S*)-ketol was isomerized to its five-membered isomer. Moreover, when submitted to appropriate culture conditions, the racemic ketol afforded the (*S*)-lactone (81 % ee) as well as the unchanged (*R*)-lactol of 97 % ee The remaining substrate could then be further treated by *m*-chloroperbenzoic acid to afford the (*R*)-hydroxylactone enantiomer.

Extensive studies have been performed on the microbial Baeyer-Villiger oxidation of bicyclic [3.2.0] ketones and analogues. These studies were prompted by the important findings of Furstoss and coworkers[100], who determined that the oxygenation of bicyclo[3.2.0]hept-2-en-6-one using *Acinetobacter* sp. TD 63 led to *two* regioisomeric lactones in equal quantities and almost quantitative yield. The first arises from the "normal" oxygen insertion mode into the more substituted carbon-carbon bond, whereas the second is the result of an oxygen insertion into the less substitiuted bond leading to the so-called "abnormal" lactone. Moreover, both these lactones were of high optical purity i. e. showing a 98 % ee for the (–)-(1*S*, 5*R*) isomer and a 95 % enantiomeric excess for the (–)-(1*R*, 5*S*) enantiomer. These results appeared to suggest that biological Baeyer-Villiger oxidations could indeed be used for the large-scale preparation of optically active lactones.

In the case of bicyclo[3.2.0]hept-2-en-6-one, each one of the substrate enantiomers reacts with a different and divergent regioselectivity for the oxygen atom insertion. This result is noteworthy since it describes for the first time such an almost perfect

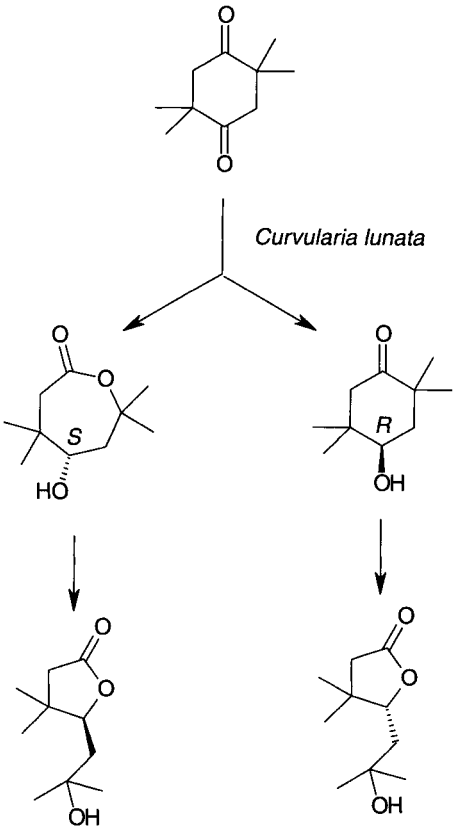


Figure 16.5-21. Baeyer-Villiger oxidation of 2,2,5,5-tetramethyl-1,4-cyclohexane-dione by *Curvularia lunata*.

regio- vs. enantioselectivity for the Baeyer-Villiger oxygenation. A more complete study[101], aimed at exploring the synthetic potential of these reactions, confirmed that this *enantiodivergent* selectivity is not restricted to one particular substrate but is a general phenomenon within a series of similar compounds. Two strains of bacteria, *Acinetobacter* sp. TD 63 and *A. calcoaceticus* NCIMB 9871 were used throughout this study and led to almost identical results. In most cases, both "normal" and "abnormal" lactones were obtained in approximately 1:1 ratios and with almost quantitative yields. Also, it was observed as shown in Fig. 16.5-22 that the "abnormal" lactone, which is not accessible using conventional Baeyer-Villiger oxidation, always shows very high ee values, whereas the enantiomeric purity of the "normal" lactone is somewhat lower for the substrate bearing a saturated six-membered ring. Both of these lactones are interesting chiral synthons; the "normal" one being an important chiron for prostaglandin synthesis. It is noteworthy that all lactones of a particular type are formed from the same enantiomer of the starting ketone: thus, the substrate enantiomer bearing an (*S*)-configuration at the bridge-head carbon atom α to the carbonyl group leads to the "normal" lactones, whereas the (*R*)- configuration affords the "abnormal" ones.

Similar results were obtained in the course of a study conducted on bicyclic

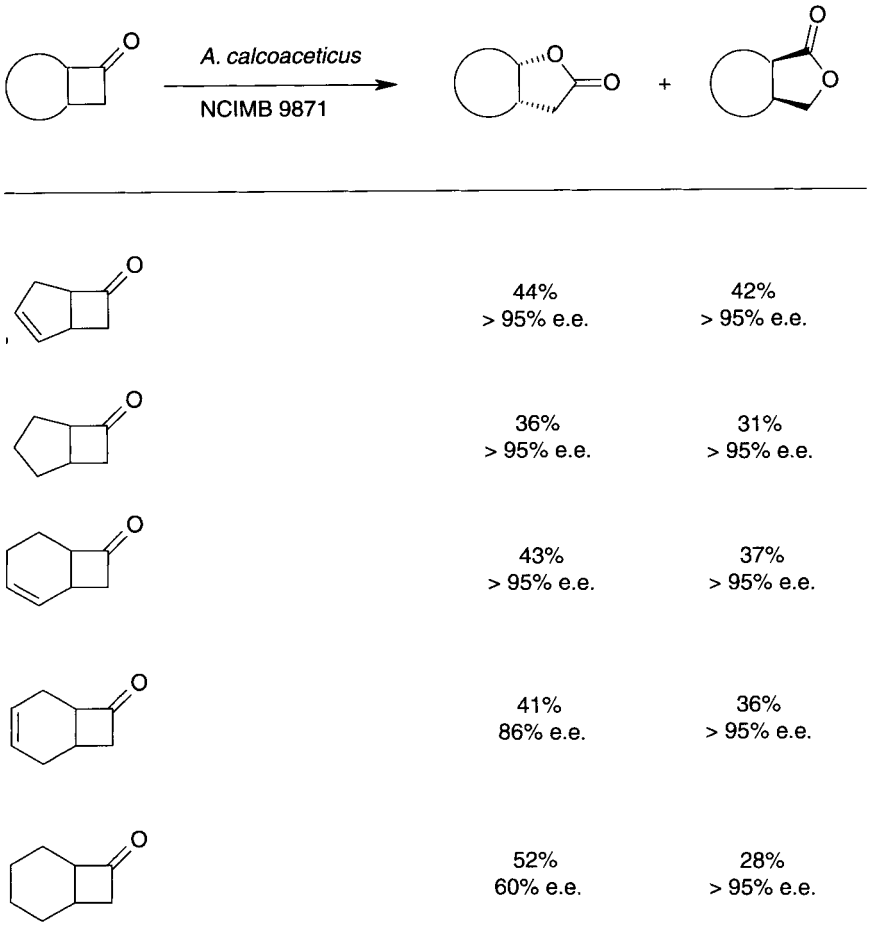


Figure 16.5-22. Oxidation of various [*n*.2.0] bicyclic ketones with *Acinetobacter calcoaceticus* NCIMB 9871.

substrates bearing an oxygen atom in the five or six-membered ring[102] (Fig. 16.5-23). Here again, equivalent ratios as well as high ee values were obtained for both the 'normal' and "abnormal" lactones. Since the lactones are unreported in the literature in their optically inactive form, detailed studies using circular dichroism were conducted in order to attribute the absolute configuration of the products.

Whilst the whole-cell approach has proved invaluable, the associated problems of overmetabolism and side reactions can be encountered. Another way to counter the problems of high cost in using isolated BVMOs is to use an NADH dependent enzyme, as NADH retails at approximately one tenth of the cost of NADPH. The Type 2 DKCMOs from *Pseudomonas putida* ATCC 17453 (≡ NCIMB 10007) are NADH dependent, and Grogan et al. were successful in applying a complement of these enzymes, termed MO1, to the transformation of bicyclo[3.2.0]hept-2-en-6-one, to yield another enantiodivergent mix of lactones enantiomeric to those obtained

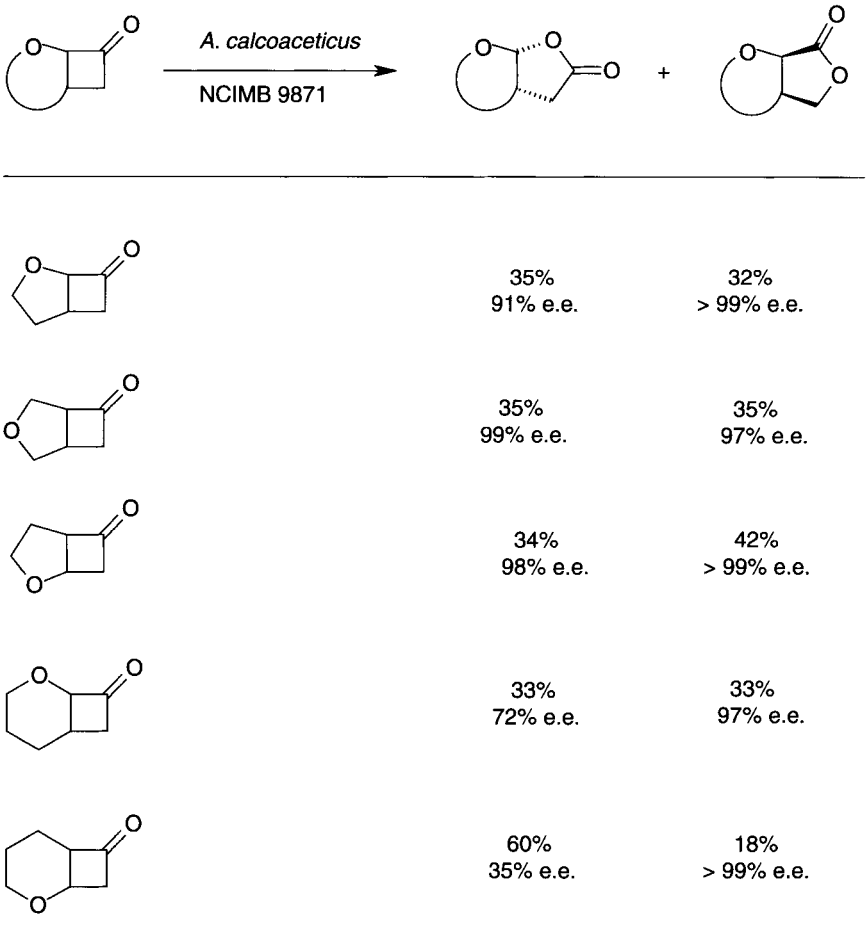
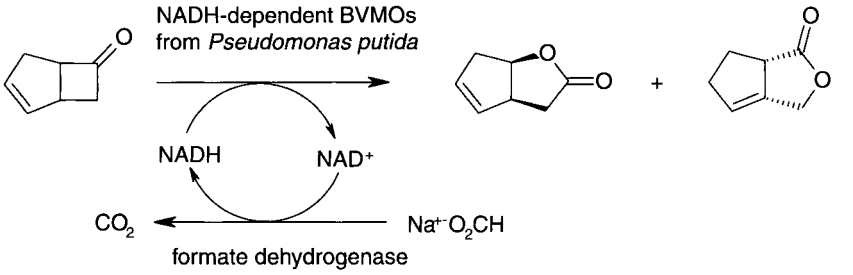


Figure 16.5-23. Oxidation of various oxo-[*n*.2.0] bicyclic ketones with *Acinetobacter calcoaceticus* NCIMB 9871.

with *A. calcoaceticus* NCIMB 9871/TD 63. The use of NADH dependent enzymes is also important in this context, as it allows use of the NAD dependent formate dehydrogenase/sodium formate recycling strategy for cofactor regeneration[103], reducing costs still further. Interestingly, the separated isoenzymes, 2,5-diketocamphane 1,2-monooxygenase and 3,6-diketocamphane 1,6-monooxygenase were shown to have different selectivities for this transformation, compromising the result obtained with MO1[104] (Fig. 16.5-24). Further transformations of this ketone by luminescent bacteria containing NADH dependent luciferases (also Type 2 BVMOs) have also been reported[105], although characterization of cell-free systems employing these enzymes has not been investigated further.

The biotransformation of bicyclo[3.2.0]hept-2-en-6-one using whole cell suspensions of the fungus *Cylindrocarpon destructans* gave not only different ratios of both lactones depending on the degree of conversion, but also no enantioselectivity was



'MO1'	63%, 60% e.e.	37%, 95% e.e.
2,5-DKCMO	57%, 82% e.e.	43%, 100% e.e.
3,6-DKCMO	17%, 10% e.e.	13%, 72% e.e.

Figure 16.5-24. Biotransformation of bicyclo[3.2.0]hept-2-en-6-one by NADH dependent BVMOs from camphor grown *Pseudomonas putida* ATCC 17453.

observed[106]. Further fungal biotransformations described by Carnell and Willetts showed that a series of dematiaceous fungi were also able to lactonize the same substrate[107]. These included various *Curvularia* and *Dreschlera* species. Some of these fungi produced both regioisomeric lactones with a high degree of stereoselectivity, whilst others produced mostly the 3-oxa lactone. The test strains of *Curvularia lunata* and *Dreschlera australiensis* gave lactones with equal and almost opposite degrees of regio- and stereoselectivity. Importantly, the biotransformation of bicyclo[3.2.0]hept-2-en-6-one by another fungus, *Cunninghamella echinulata* NRRL 3655, is unique in that it results in a *resolution* of the parent substrate to yield only the "abnormal" (–)-(1*R*, 5*S*)-3-oxa lactone in 30% yield and 95% ee[108]. This chiral synthetic intermediate has been used to synthesize both single enantiomer cyclosarkomycin[108] and the marine brown algae pheremones (+)-multifidene and (+)-viridiene[109] (Fig. 16.5-25).

Further reports by Furstoss and coworkers concerned Baeyer-Villiger oxidation of α-substituted cyclopentanones[110]. Using the same two *Acinetobacter* strains used previously, this study aimed to explore the possibility of synthesising optically active δ-lactones bearing aliphatic chains, these compounds being of particular interest as chiral synthons. This study showed that various lactones of (*S*) configuration can be obtained in fair yields with moderate to excellent ee values depending on the chain length and on the conversion ratio. Using *Acinetobacter calcoaceticus* NCIMB 9871 it was, however, necessary to run these biotransformations in the presence of tetraethylpyrophosphate (TEPP), a well known inhibitor of hydrolases. This was necessary in order to avoid hydrolytic degradation of the δ-lactones formed. The use of this inhibitor was, however, unnecessary when using the *Acinetobacter* sp. TD 63 strain which is known to lack a lactone hydrolase. One interesting application of this study was the preparative two-step synthesis of both enantiomers of 5-hexadecanolide, a

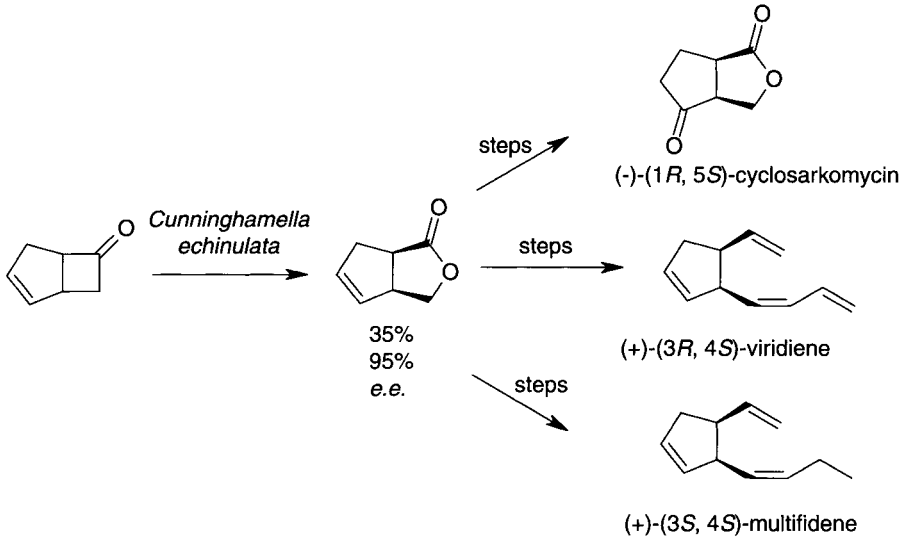


Figure 16.5-25. Biotransformation of bicyclo[3.2.0]hept-2-en-6-one by *Cunninghamella echinulata* NRRL 3655 and synthetic targets.

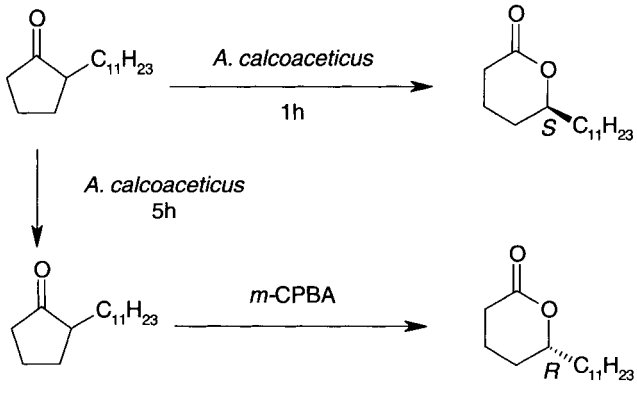
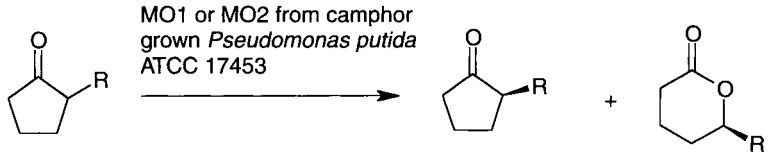


Figure 16.5-26. Baeyer-Villiger oxidation of α-undecylcyclopentanone: synthesis of either enantiomer of hexadecanolide.

pheromone isolated from the oriental hornet *Vespa orientalis*. As shown in Fig. 16.5-26, Baeyer-Villiger oxidation of racemic undecylcyclopentanone with *A. calcoaceticus* NCIMB 9871 led to a 25% isolated yield of (*S*)-5-hexadecanolide showing an ee of 74%. Interestingly, a 30% yield of remaining (*R*)-2-undecylcyclopentanone of 95% optical purity can also be isolated using a longer incubation time, thus allowing direct access, via chemical Baeyer-Villiger oxidation, to the (*R*)-(+)-5-hexadecanolide known to be the sole bioactive enantiomer.

The biotransformation of α-substituted cycloalkanones using the BVMOs from camphor grown *Pseudomonas putida* has also been investigated in depth. Whilst the NADPH dependent activity corresponding to 2-oxo-Δ^3-4,5,5-trimethylcyclopentenylacetyl-Co-A monooxygenase (and termed MO2) resolved a series of α-alkyl cyclopentanones with good selectivity, poorer resolution of these compounds was per-

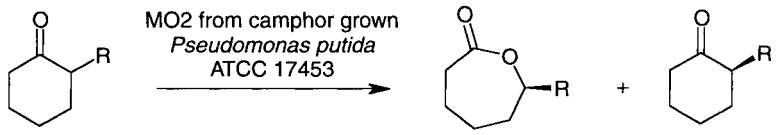


MO1

R	Yield ketone	e.e. ketone	Yield lactone	e.e. lactone
C_4H_9	14	9	16	58
C_6H_{13}	48	48	34	74
C_8H_{17}	35	22	11	90

MO2

R	Yield ketone	e.e. ketone	Yield lactone	e.e. lactone
C_4H_9	26	-	40	95
C_6H_{13}	51	75	35	92
C_8H_{17}	44	59	29	95



MO2

R	Yield ketone	e.e. ketone	Yield lactone	e.e. lactone
C_6H_{13}	30	65	36	72
C_8H_{17}	49	61	34	77
CH_2CO_2Et	43	89	30	93
CH_2CH_2OAc	13	75	34	83

Figure 16.5-27. Biotransformation of 2-substituted monocyclic ketones by BVMOs from camphor grown *Pseudomonas putida* ATCC 17453.

formed by the NADH dependent MO1 complement[104] (Fig. 16.5-27). An extension to this study revealed that MO2 could be used to resolve a series of α-substituted cyclohexanones wherein the subsituents consisted of esters, acetates and common protecting groups[111]. This led to the development of a chemoenzymatic synthesis of (*R*)-(+)-lipoic acid incorporating a BVMO catalyzed resolution as the key step (Fig. 16.5-28). Interestingly, the preferred selectivity of cyclopentanone monooxygenase from *Pseudomonas* sp. NCIMB 9872, is opposite to that of MO2, and in a

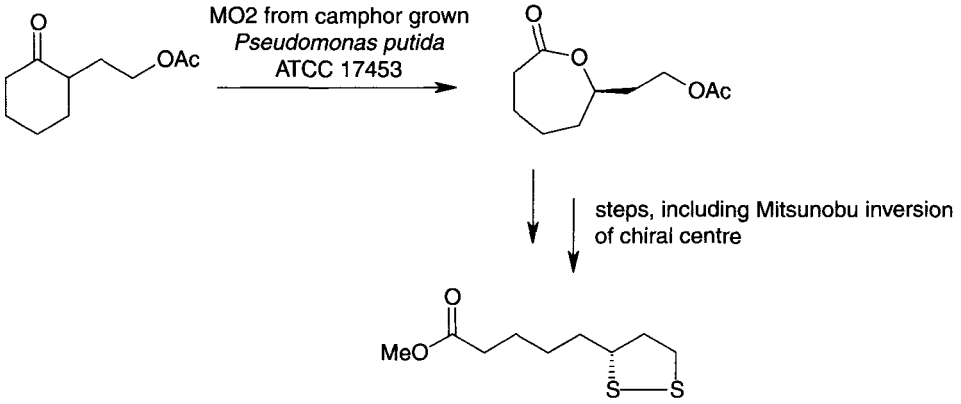


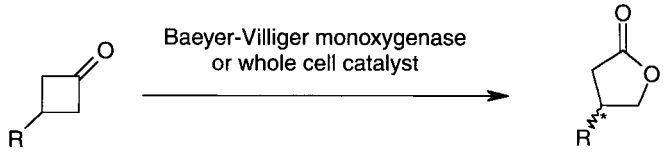
Figure 16.5-28. Chemoenzymatic synthesis of (+)-lipoic acid incorporating a BVMO catalysed resolution as the key step.

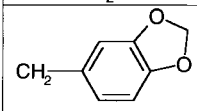
separate investigation, it was suggested that this enzyme be used in the place of MO2 to eliminate the need for the Mitsonobu inversion in the chemoenzymatic synthesis[112].

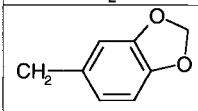
The biological Baeyer-Villiger oxidation has also been applied, in a variety of forms, to the production of optically active lactones from prochiral 3-substituted cyclobutanones. A series of cyclobutanones was subjected to oxidation by *Acinetobacter* sp. and to the MO1 and MO2 enzyme preparations derived from camphor-grown *Pseudomonas putida* ATCC 17453[113]. The results are summarized in Fig. 16.5-29. In general, the reactions performed with *Acinetobacter* sp. displayed better enantioselectivities, but the value of a multi-biocatalyst approach was illustrated by the fact that certain BVMOs from *P. putida* displayed opposite enantioselectivity. A further series of cyclobutanone substrates was oxidized by *Acinetobacter* sp. and by the fungus *Cunninghamella echinulata*[114] (Fig. 16.5-30). The lactonization of 3-(4'-chlorobenzyl)-cyclobutanone was performed by this fungus to yield (*R*)-lactone of 99% ee in 30% yield, which was used in a chemoenzymatic synthesis of baclofen[115], a lipophilic derivative of γ-aminobutyric acid. The *Cunninghamella* strain was also used to oxidize 3-(benzyloxymethyl)-cyclobutanone to the optically pure (*R*)-(-)-γ-butyrolactone, which was used in enantiodivergent chemoenzymatic syntheses of (*R*)- and (*S*)-proline[116] .

The oxidation of either enantiomer of menthone and dihydrocarvone by *Acinetobacter* sp. were also reported[117]. (–)-Menthone is not metabolized but (+)-menthone leads to the expected lactone, whereas both enantiomers of dihydrocarvone are oxidized. Thus (–)-dihydrocarvone leads to the expected lactone, whereas (+)-dihydrocarvone afforded the unexpected 'abnormal' lactone product (Fig. 16.5-31). Both enantiomers of dihydrocarvone are also transformed by MMKMO[51] from *Rhodococcus erythropolis* DCL 14, which in contrast to *Acinetobacter* sp., also transforms both enantiomers of menthone.

Taschner and coworkers described the oxidation of *cis*-3,5-dimethylcyclohexanone by whole-cell preparations of *A. calcoaceticus* NCIMB 9871[118], which led directly to



***A. calcoaceticus* NCIMB 9871**			
R	Conversion	Yield lactone	e.e. lactone
Bu	95	68	(*S*)-,17%
Bu^i	98	56	(*R*)-, 84%
CH_2Ph	100	57	(*R*)-, 82%
	100	83	(*R*)-, 95%
CH_2OCH_2Ph	100	89	(*S*)-, 55

MO1			
R	Conversion	Yield lactone	e.e. lactone
Bu	100	nd	(*R*)-, 69
Bu^i	78	nd	(*R*)-, 91
CH_2Ph	58	40	(*S*)-, 15
	48	38	(*R*)-, 7
CH_2OCH_2Ph	98	74	(*S*)-, 74

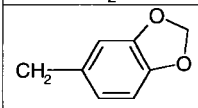
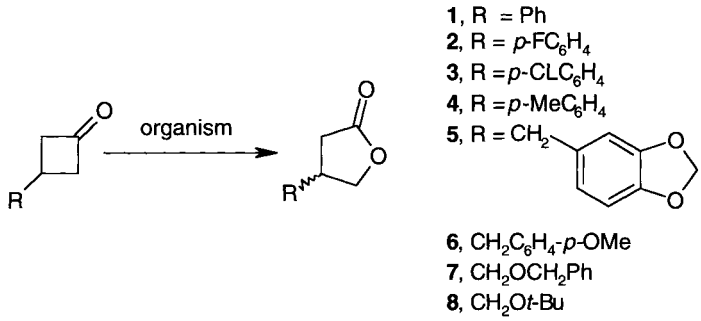
MO2			
R	Conversion	Yield lactone	e.e. lactone
Bu	93	nd	(*R*)-, 54
Bu^i	97	nd	(*R*)-, 85
CH_2Ph	37	26	(*S*)-, 20
	71	6	(*S*)-, 14
CH_2OCH_2Ph	95	nd	(*R*)-, 90

Figure 16.5-29. Biotransformation of prochiral 3-substituted cyclobutanones using BVMOs.

the corresponding optically active lactone and thence to the hydroxyacid, which was converted into the methylester by reaction with diazomethane. This methylester, which was shown to be optically active, is a key intermediate in the synthesis of the polyether antibiotic ionomycin.

In addition, several bridged bicyclic compounds have been examined as potential substrates (Fig. 16.5-32). In contrast to the regiodivergent behaviour of the [*n*.2.0] bicyclic compounds, in these cases, only one lactone product is usually obtained. This high selectivity compares favorably with the chemical Baeyer-Villiger oxidation of compounds of this type, which often afford regiomixtures[119]. In addition, the



Ketone	Microorganism	Yield	e.e.
1	*C. echinulata*	65	(*R*)-, 98
	A. calcoaceticus	70	(*R*)-, 43
	Acinetobacter TD63	84	(*R*)-, 47
2	*C. echinulata*	80	98
	A. calcoaceticus	89	19
	Acinetobacter TD63	92	5
3	*C. echinulata*	30	(*R*)-, 98
	A. calcoaceticus	88	(*S*)-, 85
	Acinetobacter TD63	15	(*S*)-, 89
4	*C. echinulata*	4	nd
	A. calcoaceticus	73	(*S*)-, 91
	Acinetobacter TD63	61	(*S*)-, 93
5	*C. echinulata*	68	(*S*)-, 91
	A. calcoaceticus	70	(*S*)-, 100
	Acinetobacter TD63	64	nd
6	*C. echinulata*	68	(*S*)-, 91
	A. calcoaceticus	83	(*S*)-, 96
	Acinetobacter TD63	94	(*S*)-, 94
7	*C. echinulata*	74	(*R*)-, 98
	A. calcoaceticus	89	(*S*)-, 55
	Acinetobacter TD63	90	(*R*)-, 25
8	*C. echinulata*	25	98
	A. calcoaceticus	43	89
	Acinetobacter TD63	15	88

Figure 16.5-30. Biotransformation of prochiral cyclobutanones by three whole-cell preparations.

obtained bridgehead lactones are often described to be of high optical purity. The benzyloxy derivative is known to be an important intermediate for prostaglandin synthesis. The residual fluorinated bicyclic ketone of high enantiomeric excess was used to synthesize an antiviral carbocyclic nucleoside[120]. In this last case, detailed studies showed that the first formed product is the corresponding alcohol (about 80% conversion) and that over the next 3 h period, the alcohol concentration decreased, the amount of ketone rose and the production of lactone started[121]. This observation led to an elegant closed-loop recycling procedure, as shown in Fig. 16.5-33, where the alcohol dehydrogenase from *Thermoanaerobium brockii* was used in conjunction with the purified monooxygenase from *A. calcoaceticus* NCIMB 9871. In

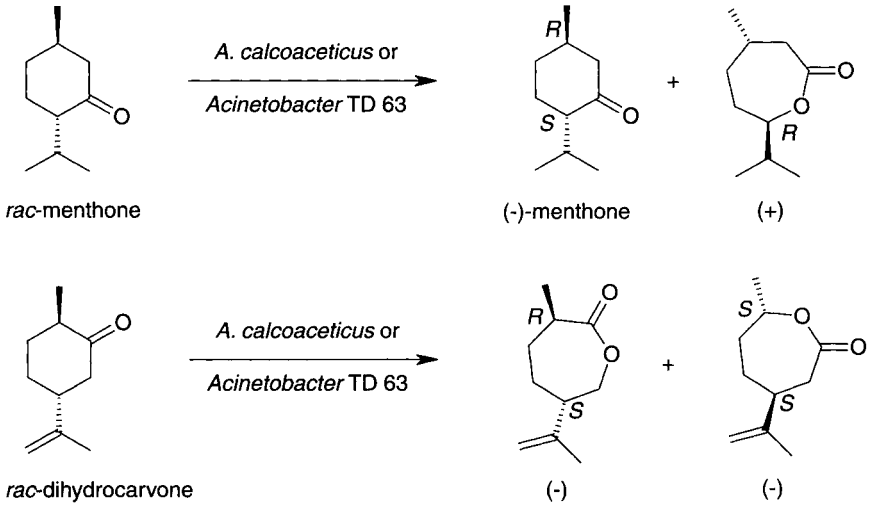


Figure 16.5-31. Oxidation of dihydrocarvone enantiomers with *Acinetobacter calcoaceticus* NCIMB 9871 and *Acinetobacter* sp. TD63.

this case, the substrate alcohol also serves as a co-substrate for the NADPH recycling reaction. Thus, *endo*-bicyclo[2.2.1]heptan-2-ol was transformed using catalytic amounts of NADP. An analogous recycling loop was set up using the NAD dependent alcohol dehydrogenase from *Pseudomonas* sp. NCIMB 9872 and the NADH dependent MO1 isozyme complement from *Pseudomonas putida* ATCC 17453, for the oxidation of 7- *endo*-methylbicyclo[3.2.0]hept-2-en-6-ol [122].

A further series of prochiral bicyclic [2.2.1] substrates have also been studied by Taschner and coworkers and lead generally to lactones of high enantiomeric purity. One of these is a valuable precursor for chorismic acid synthesis [97].

The transformation of a series of norbornanone derivatives (Fig. 16.5-34) was studied by Roberts and coworkers who determined that both the MO1 complement of NADH dependent BVMOs from *Pseudomonas putida* ATCC 17453 and the NADPH dependent fraction MO2 were successful in the resolution of hydroxy, acetoxy and benzyloxy norbornanones [123]. Interestingly 25DKCMO and 36DKCMO when separate, displayed notably different reactivity toward the hydroxy and acetoxy derivative, again emphasizing their complementary nature as potential individual biocatalysts. The benzyloxy lactone is an intermediate in the synthesis of the insect antifeedant azadirachtin.

Further studies also been performed on the bicyclo[3.2.0]heptan-6-one series of compounds [124, 125]. These results are summarised in Fig. 16.5-35. Oxidation of this ketone with *Pseudomonas* NCIMB 9872 gave the (1*S*, 5*R*)-lactone of low optical purity (23% ee) with only small amounts (5%) of the isomeric lactone, whereas its oxidation with an *Acinetobacter* sp. gave these lactones in a 9:1 ratio and a modest yield, a result quite different from the one described previously. However, oxidation of 7-*endo*-methylbicyclo[3.2.0]hept-2-en-6-one using either *Pseudomonas* sp. or *Acinetobacter* sp. produced optically pure (ee > 96%) of both lactones in equal quantities

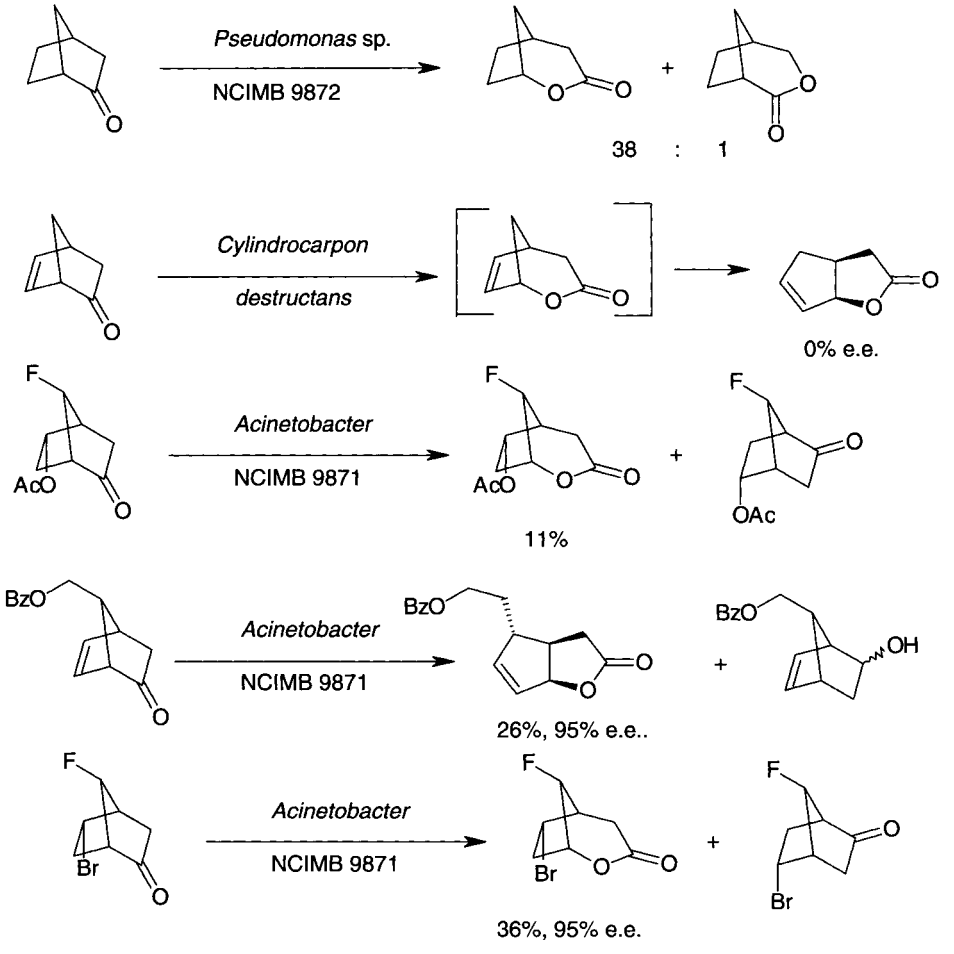


Figure 16.5-32. Baeyer-Villiger oxidation of various [2.2.1] bicyclic substrates.

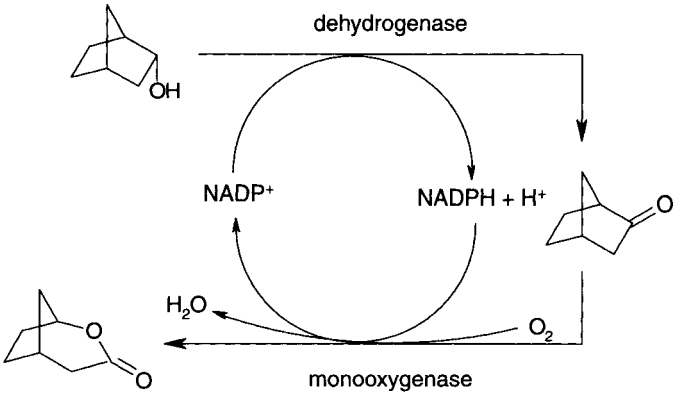
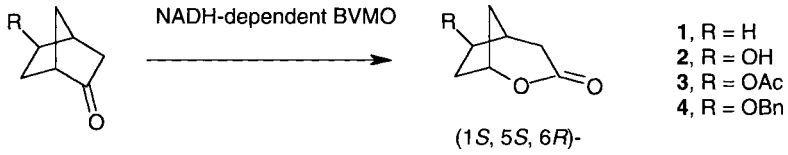


Figure 16.5-33. Closed-loop recycling procedure for NADPH recycling using the substrate alcohol as the reducing agent.



Enzyme	Substrate	Conversion (%)	Lactone e.e. (%)
25DKCMO	**1**	20	60
36DKCMO	**1**	48	>90
25DKCMO	**2**	0	-
36DKCMO	**2**	33	>95
25DKCMO	**3**	35	>95
36DKCMO	**3**	0	-
'MO1'	**4**	39	>95

Figure 16.5-34. Biotransformation of norbornanone derivatives using NADH dependent BVMOs from camphor grown *Pseudomonas putida* ATCC 17453.

(combined yields 50–55%). Surprisingly, 7,7-dimethylbicyclo[3.2.0]hept-2-en-6-one was oxidized by the *Acinetobacter* strain to give exclusively one lactone of 29% ee, a very low enantioselectivity. The bromohydrin obtained from this substrate led to similar results, yielding the same type of oxidation. This can be considered as being the "normal" lactone since substitution with two methyl groups makes this carbon-carbon bond the more substituted one. Again, the MO1 isozymic complement from *Pseudomonas putida* was successful in generating the complementary enantiomers from *endo*-methyl and dimethyl derivatives with good enantiomeric excess [103].

16.5.4
Models for the Action of Baeyer-Villiger Monooxygenases

The results of biological Baeyer-Villiger oxidations have been, in some cases unpredictable and surprising, and, in the continued absence of a structure of one of these enzymes, several groups have attempted to explain the various observations of selectivity with an increasingly complex series of models.

Initially, some workers proposed that enantiodivergent biotransformations of the type witnessed in the oxygenation of bicyclo[3.2.0]hept-2-en-6-one by, for instance CHMO and 25DKCMO could be due to the presence in either of these preparations of two separate enzymatic activities. Whilst this was once and indeed still is, a reasonable assumption in the light of results obtained with whole-cell preparations, the use of highly purified preparations of the two named enzymes to effect this biotransformation [104, 126] have eliminated this possibility in these cases. The phenomenon of enantiodivergence has therefore been addressed with respect to one enzyme active site.

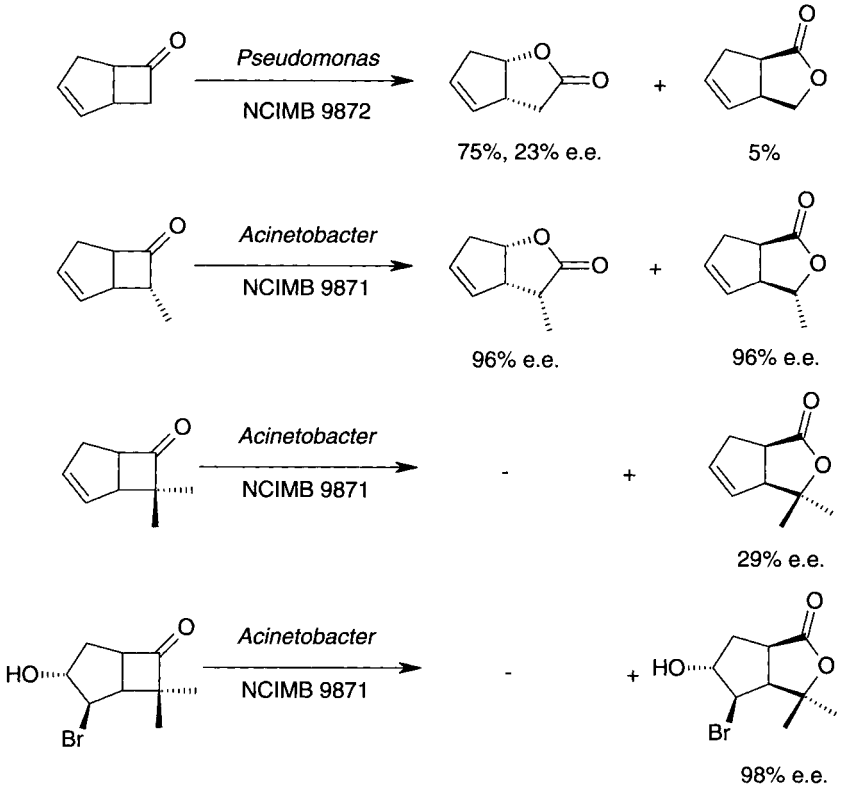


Figure 16.5-35. Baeyer-Villiger oxidation of various [*n*.2.0] bicyclic compounds.

The first model was proposed by Furstoss and coworkers, based on steric and stereoelectronic considerations. In this model, shown in Fig. 16.5-36, the 4-*a*-hydroxyperflavin is considered as being the oxygen transfer agent, according to the hypothesis of Walsh and coworkers [84]. The enantioselectivity of the reaction would be due to a different positioning of each intermediate in the active site. It is supposed, primarily, that the attack of the hydroperoxyflavin should take place on the least hindered face of the ketone. On the other hand, the migrating C-C bond of the peroxidic intermediate should be antiperiplanar to the peroxidic bond and to a non-bonded electron pair of the hydroxide group, as suggested for chemical Baeyer-Villiger oxidations. Thus, the cycloalkyl part of the (*S*,*S*)-enantiomer of the ketone (the one leading to the "normal" lactone) could be accommodated in only one region of the active site (position 1). Position 2 would never be adopted due to some steric hindrance with the active site (dotted cube). Similarly, in the case of the (*R*,*R*)-enantiomer, position 4 would be favored over position 3 leading to the "abnormal" lactone. This model was augmented by further work by the inclusion of results obtained with both monocyclic monterpene [117], 3-substituted cyclobutanone substrates [113] and α-substituted cyclohexanones [127].

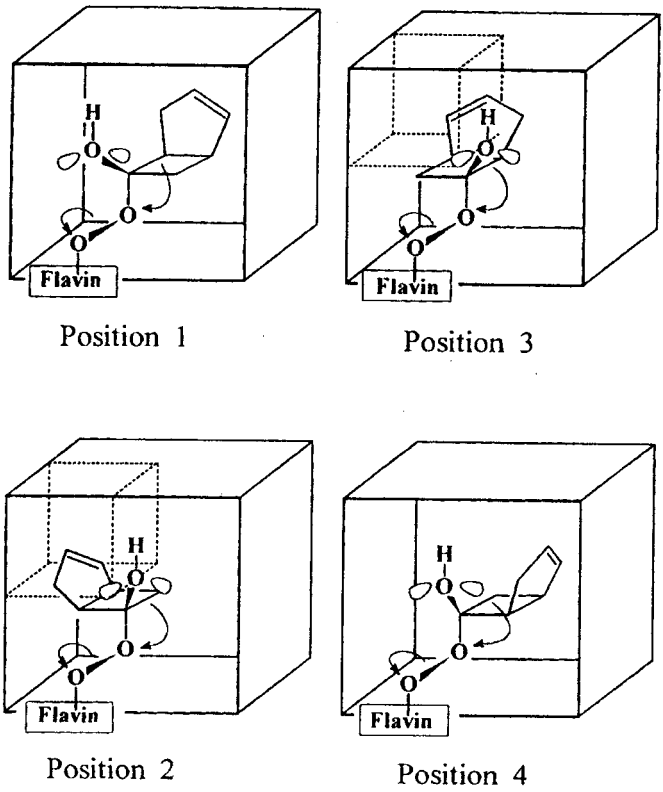


Figure 16.5-36. Furstoss model for the active site of cyclohexanone monooxygenase from *Acinetobacter calcoaceticus* NCIMB 9871.

Taschner and coworkers proposed a similar model based on two other flavoenzymes; the human and *E. coli* glutathione reductase. The FAD binding domain of glutathione reductase and *p*-hydroxybenzoate hydroxylase have been shown to resemble each other closely via comparison of their respective X-ray crystal structures. Extrapolating this information to CHMO leads to the proposal that the hydroperoxide is attached to the *re*-face of the isoalloxazine ring and that the ketone substrates approach the hydroperoxide from the direction of the dimethylbenzene moiety[97]. Further stereochemical and stereoelectronic considerations lead to a hypothesis explaining the observed stereoselectivities.

In the model of Furstoss and coworkers, stereoselectivity of CHMO is determined by the differentiation of groups of different sizes in the active site. A different model, proposed by Kelly and coworkers[128–130], extends Taschner's idea that the source of stereoselectivity might be the flavin cofactor itself. It was suggested that the stereoselectivity of oxygen insertion arises solely as a result of the flavin face, *re*- or *si*-, from which the hydroperoxide attacks. This would lead to two distinct Criegee intermediates of opposing absolute configuration (Fig. 16.5-37). Hence it was

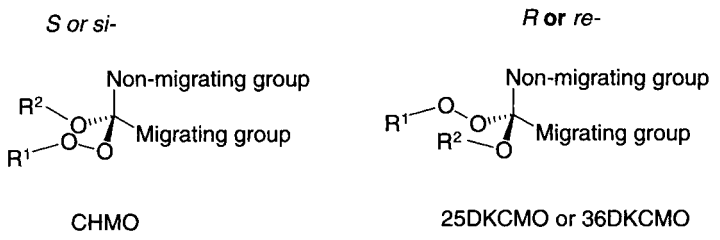


Figure 16.5-37. Schematic representation of enantiomeric Criegee intermediates for the enzymatic Baeyer-Villiger reaction.

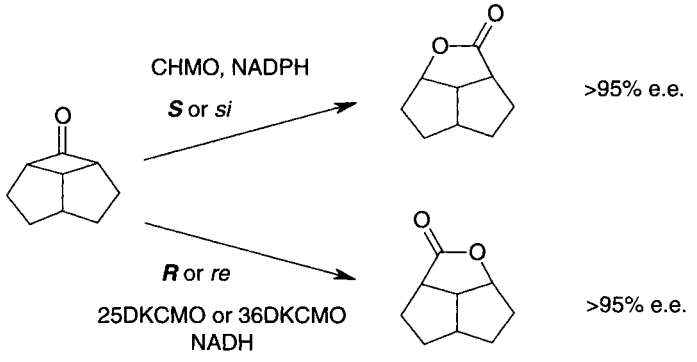


Figure 16.5-38. Enantioselective Baeyer-Villiger oxidation of a tricyclic ketone by Type 1 and Type 2 BVMOs.

demonstrated that for the tricyclic ketone shown in Fig. 16.5-38 for which attack from only the *exo*-face is possible, pure preparations of BVMOs always resulted in lactones of >95 % ee Interestingly, all Type 1, FAD plus NADPH dependent BVMOs yield lactone from the (*R*)-configuration of the intermediate, and all Type 2, NADH plus FMN dependent BVMOs yield lactone from the (*R*)-intermediate. Substrate interaction with the topology of the active site must also be considered however, as the enantiocomplementary DKCMOs, both proposed to catalyze oxygen insertion via (*R*)-Criegee intermediates, catalyze complementary resolutions of racemic camphor[67].

This additional dependence on active site topology for selectivity in CHMO was carefully considered by Ottolina et al.[131], who developed a sophisticated cubic space model for the active site of CHMO (Fig. 16.5-39). This group was able to show that, for example, for the biotransformation of 7-*endo*-methylbicyclo[3.2.0]hept-2-en-6-one, of the eight possible intermediates in oxidation, the only two "allowed" by the model were the two which led to the lactones observed by experiment. The model was successfully applied to a series of other ketones and also predicts the stereoselectivity of sulfur oxidation by this enzyme[132]. The group of Colonna established in a series of reports that CHMO was able to catalyze the oxidation of a range of alkylaryl sulfides, benzyl alkyl sulfides, functionalized sulfides and 1,3-dithioacetals with absolute configuration and enantiomeric excesses being highly dependent

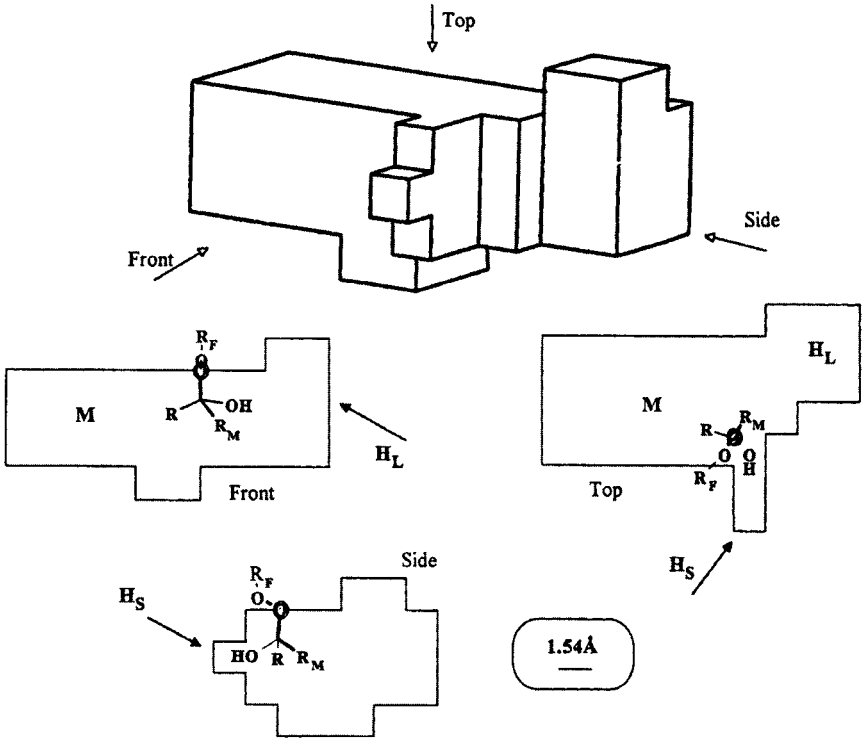


Figure 16.5-39. Cubic space filling model of the active site of cyclohexanone monooxygenase from *Acinetobacter calcoaceticus* NCIMB 9871, based on the results of the oxidations of a series of bicyclic ketones. The catalytic oxygen is circled. The main (M) hydrophobic large (H_L) and hydrophobic small (H_S) pockets are depicted. The correct arrangements of the Criegee intermediate are also shown.

on the structure of the substrate[93]. This group has also recently reported the first asymmetric oxidation of tertiary amines using CHMO[133].

The ability of BVMOs to oxidize sulfur was also exploited by Beecher and Willetts in order to construct space filling cubic models of the active site of the DKCMO enzymes from *Pseudomonas putida* ATCC 17453 (Fig. 16.5-40). They note that the more relaxed enantiospecificity of 36DKCMO, at least in terms of sulfoxidation, appears to be due to an overall larger 3D cubic space available in the active site[134]. 36DKCMO appears to be the best candidate for a first X-ray structure of a BVMO, as preliminary crystal data have been reported[88].

16.5.5
Conclusion and Outlook

It is apparent from the many application of BVMOs in synthesis, that these enzymes currently represent the most valuable method of effecting the enantioselective

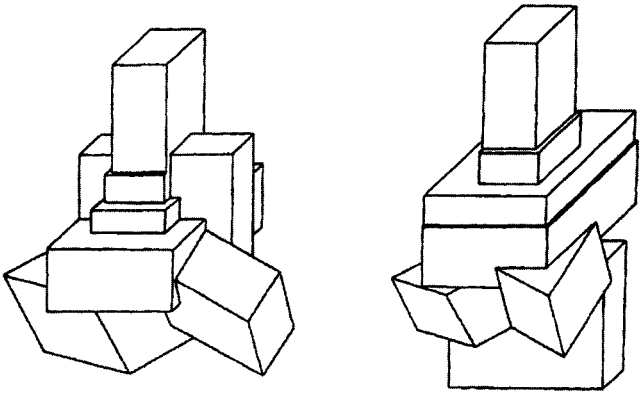
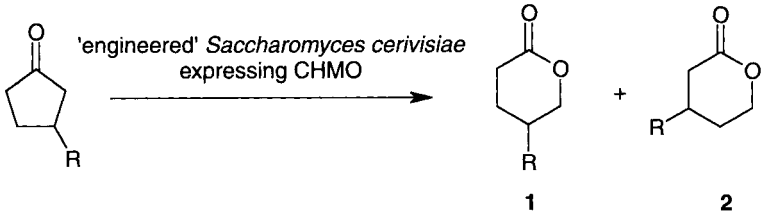


Figure 16.5-40. Cubic space filling models of active sites of: right, 3,6-diketocamphane 1,6-monooxygenase; and left, 2,5-diketocamphane, 1,2-monooxygenase based on results of sulfoxidations of a series of sulfide substrates.



R	Ratio **1**:**2** Combined yield lactones	e.e. lactone **1** (%)	e.e. lactone **2** (%)
Me	13:87 95%	9	36
Et	80:20 80%	33	19
n-Pr	83:17 44%	33	60
n-Bu	99:1 34%	38	-
n-Oct	99:1 19%	16	-

Figure 16.5-41. Biotransformation of 3-alkylcyclopentanones by "engineered" *Saccharomyces cerivisiae* expressing CHMO.

Baeyer-Villiger reaction. The primary sources of BVMO enzymes carry associated disadvantages that must now be addressed, although recent biotechnological advances suggest that BVMOs will be more accessible to the synthetic organic chemist in the future.

Type 1 BVMOs

1	Steroid monooxygenase		
	Cylindrocarpon radicicola	2	**A**-E-W-A-E-E-**F**-**D**-**V**-**L**-**V**-V-**G**-**A**-**G**-A-**G**-**G**-
2	CHMO		
	Rhodococcus coprophilus	2	**A**-Q-T-I-H-G-V-**D**-**A**-**V**-**V**-**I**-**G**-**A**-**G**-**F**-**G**-**G**-I-**Y**-**A**-**V**-H-**K**-
3	CHMO		
	Acinetobacter NCIMB 9871	1	M-**S**-Q-L-M-D-**F**-**D**-**A**-I-**V**-**I**-**G**-**G**-**G**-**F**-**G**-**G**-L-**Y**-**A**-**V**-K-**K**-
4	CPMO		
	Pseudomonas NCIMB 9872	14	-N-**S**-V-N-D-K-L-**D**-**V**-**L**-L-**I**-**G**-**A**-**G**-**F**-
5	Steroid monooxygenase		
	Rhodococcusrhodochrous	1	M-N-G-Q-H-P-R-**V**-**V**-**V**-A-A-P-D-A

Type 2 BVMOs

6	2,5-DKCMO		
	Pseudomonas putida	1	-**M**-Q-A-**G**-F-F-G-T-**P**-**Y**-D-L-**P**-T-R-T-**A**-R-Q-M-
7	3,6-DKCMO		
	Pseudomonas putida	1	A-**M**-E-T-**G**-L-I-F-H-**P**-**Y**-M-Y-**P**-G-K-S-**A**-A-Q-

Figure 16.5-42. N-terminal amino acid sequence alignment of Type 1 BVMOs (**1–5**) and Type 2 BVMOs (**6** and **7**). Conserved residues are marked in bold.

The *Acinetobacter* strain from which CHMO is derived is a Class II pathogen as defined by the Advisory Committee on Dangerous Pathogens (ACDP), and hence, may only be handled in suitably equipped microbiological facilities. One solution to

this problem has been the cloning and expression of the gene encoding CHMO in *Saccharomyces cerivisiae*[85]. In a series of reports by Stewart and coworkers[135–137], the "designer yeast" was shown to catalyze many of the reactions which had previously been shown to be catalyzed by either whole cells of *Acinetobacter* sp. or CHMO in addition to some new ones (Fig. 16.5-41). Recently, a similar strategy has seen whole-cell preparations of *Escherichia coli* expressing recombinant CHMO for the same purpose[138]. It remains to be seen whether constraints on the use of genetically engineered microorganisms of this type will render these strains as "difficult" to manipulate as the wild-type strains.

The use of purified enzyme would circumvent the need for whole-cell containment procedures, and indeed, amounts of CHMO are now available from Fluka[139]. However, the attendant costs associated with cofactor recycling must be addressed if this approach is to prove viable. The recent production of a formate dehydrogenase suitable for use in NADP/NADPH recycling systems[140] should prove attractive in this regard, as should the further investigation of NADH dependent enzymes. The practicalities associated with the industrial scale up of biological Baeyer-Villiger reactions are currently being investigated[141].

New sources of enzyme will also become important and with the advent of genomic science, paralogs of genes that encode CHMO-like proteins are being identified amongst whole bacterial genomes, most recently those of *Pseudomonas aeruginosa*[142] and *Mycobacterium tuberculosis*[143]. The availability of gene and amino acid sequence data for BVMOs will prove useful in identifying more new activities in this manner. BVMOs of the same Type (1 or 2) exhibit sequence homology within their *N*-terminal amino acid sequences although homology between types is not conserved[130] (Fig. 16.5-42). In the future, the "tailoring" of enzyme characteristics by either rational redesign or so-called "directed" evolution approaches could also doubtless be applied to BVMOs. Fundamental to these studies would be the development of an efficient, rapid screen for BVMO activity. Rational redesign would require more knowledge of the 3D structure of these enzymes. This is one reason why the acquisition of a complete X-ray crystal structure of a BVMO must be considered of fundamental importance to the ongoing development of this area.

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16.6 Oxidation of Acids

Andreas Schmid, Frank Hollmann, Bruno Bühler

16.6.1 Introduction

At a first glance, synthetically relevant oxidations of carboxylic acids, except for oxidations at positions other than the carboxylate group, can hardly be found in literature. However, some preparative applications in whole cell catalysis were reported and will be discussed in the following (Fig. 16.6-1 A,B,C). *In vitro*, the high thermodynamic driving force for the oxidation of formate and pyruvate [E° (formate/CO_2)= -0.42 V[1]; E° (pyruvate/(acetate, CO_2)) = -0.70 V[2]] are used for the regeneration of coenzymes such as NAD(P)H or, indirectly, ATP (Fig. 16.6-1 D,E).

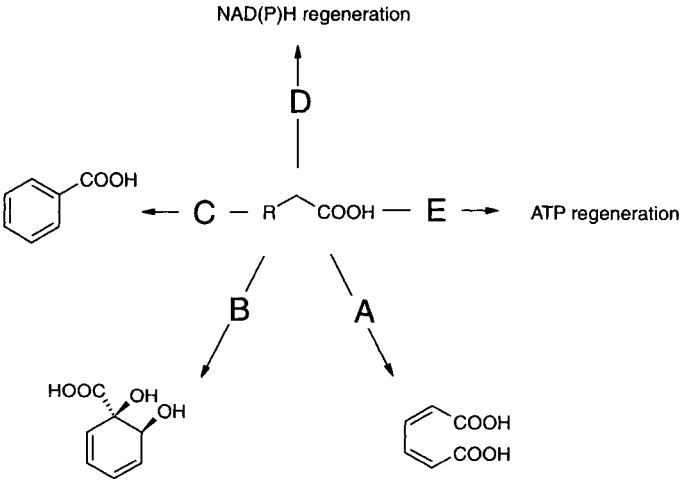

Figure 16.6-1. Synthetic and preparative applications of oxidations of acids. A, B: Oxidations of benzoic acid initiated by dihydroxylation (Sects. 16.6.4.2 and 16.6.4.3); C: oxidative decarboxylation (Sect. 16.6.4.1); D,E : energy coupling for the regeneration of coenzymes (Sects. 16.6.2, 16.6.3).

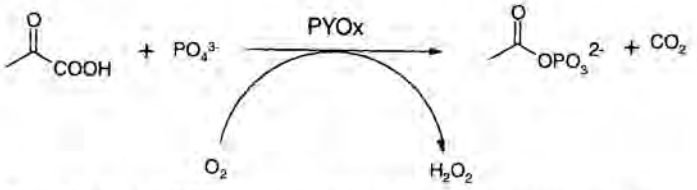

Figure 16.6-2. Oxidative phosphorylation of pyruvate by pyruvate oxidase (PYOx).

16.6.2

Pyruvate Oxidase (PYOx, E.C. 1.2.3.3)

PYOx from *Lactobacillus plantarum*[3, 4] or *Streptococcus sanguis*[5] catalyzes the decarboxylative phosphorylation of pyruvate to acetylphosphate, or the homologous arsenylation (Fig. 16.6-2).

Acetylphosphate is an important substrate for the enzyme acetate kinase (E.C. 2.7.2.1), which catalyzes the phosphorylation of various nucleotide diphosphates such as ADP, GDP, TDP, IDP, or UDP to the activated triphosphates[6–8]. This reaction can be applied to regenerate ATP in ATP-dependent enzymatic *in vitro* reactions (Fig. 16.6-3).

In a recent example, PYOx-catalyzed regeneration of ATP was coupled to *in vitro* protein biosynthesis (e.g. for human lymphotoxin)[9]. Under aerobic conditions, no external regeneration system for PYOx has to be applied; catalase however has to be added in order to destroy harmful hydrogen peroxide. An alternative to this autoregeneration approach (Fig. 16.6-3 A) was reported by Steckhan and coworkers for cases where hydrogen peroxide formation has to be prevented (Fig. 16.6-3 B)[10].

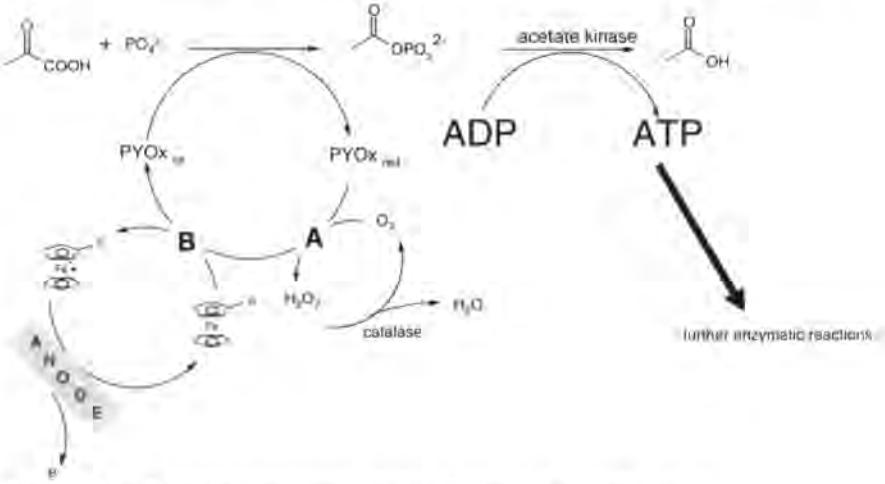

Figure 16.6-3. Decarboxylative phosphorylation of pyruvate by pyruvate oxidase as driving force for the regeneration of ATP; A: aerobic regeneration; B: indirect electrochemical regeneration.

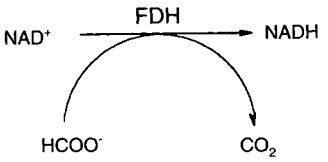

Figure 16.6-4. Regeneration of NADH using the formate dehydrogenase (FDH) reaction.

Here, the anode, together with the mediation by ferrocene, removes excess electrons from the PYOx active site.

Another possible application of the PYOx-catalyzed production of acetylphosphate lies within the *in vitro* regeneration of acetyl-CoA [11].

16.6.3
Formate Dehydrogenase (FDH, E.C. 1.2.1.2)

Probably the most prominent oxidation of a carboxylic acid is catalyzed by the enzyme formate dehydrogenase (FDH, E.C. 1.2.1.2). FDH was isolated from various bacteria, yeasts, and plants, where its physiological role is the regeneration of NADH [12].

FDH catalyzes the oxidation of formate to carbon dioxide, concomitant with the reduction of NAD^+ to NADH (Fig. 16.6-4). Because of the favorable thermodynamic equilibrium of the reaction and the volatility of the reaction product, the enzyme is commonly applied for *in situ* regeneration of NADH during asymmetric synthesis of chiral compounds [13].

FDH from *Candida boidinii* is mostly used as regeneration enzyme. It found industrial application at Degussa-Hüls AG in a leucine dehydrogenase-catalyzed reductive amination of 2-keto acids yielding various amino acids (e.g. *tert*-leucine) [14–16]. Native FDH is very selective for NAD^+. Recently a new FDH was developed by site-directed mutagenesis that shows all advantages of the NAD^+-dependent enzymes and additionally accepts $NADP^+$ as substrate [17]. The activity of the mutant with $NADP^+$ is about 60% of the wild-type FDH with NAD^+ [18].

16.6.4
Oxidations with Intact Microbial Cells

16.6.4.1
Production of Benzaldehyde from Benzoyl Formate or Mandelic Acid

Benzaldehyde can be produced from benzoyl formate with whole cells of *Pseudomonas putida* ATCC 12633 as biocatalyst [19, 20] (Fig. 16.6-5). Alternatively, but less effectively, mandelic acid can be used as starting material. A pH of 5.4 was found to be optimal for benzaldehyde accumulation. At this proton concentration, partial inactivation of the benzaldehyde dehydrogenase isoenzymes and activation of the benzoyl formate decarboxylase are reported. Fed-batch cultivation prevented substrate inhibition. *In situ* product removal is necessary to prevent product inhibition.

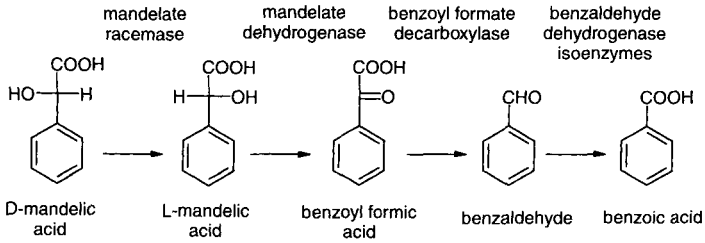

Figure 16.5-5. Degradation of tridecan-2-one with a crude cell-free preparation from a *Pseudomonas aeruginosa* strain.

Activated charcoal served as a solid-phase adsorption device [20]. Thus, benzaldehyde and thiophene-2-carboxaldehyde were obtained from benzoyl formic acid and thiophene-2-glyoxylic acid respectively, in final concentrations of up to 4.8 g L^{-1} and molar yields exceeding 85 %.

16.6.4.2
Microbial Production of *cis,cis*-Muconic Acid from Benzoic Acid

Significant effort was put into the oxidation of benzoic acid to *cis,cis*-muconic acid via a multi-step reaction catalyzed by whole microbial cells [21–24]. *Cis,cis*-muconic acid is used as raw material for the synthesis of resins and polymers (precursor of adipic acid). Furthermore, it is widely used as building block in the synthesis of pharmaceuticals and agrochemicals.

As biocatalyst, growing cells of a mutant *Arthrobacter* strain (lacking *cis,cis*-muconate derivatization activity) was used. The reaction cascade (Fig. 16.6-6) is initiated by a dioxygenation of the benzylic ring followed by decarboxylation yielding catechol, which is transformed to the product via dioxygenase-catalyzed ring cleavage.

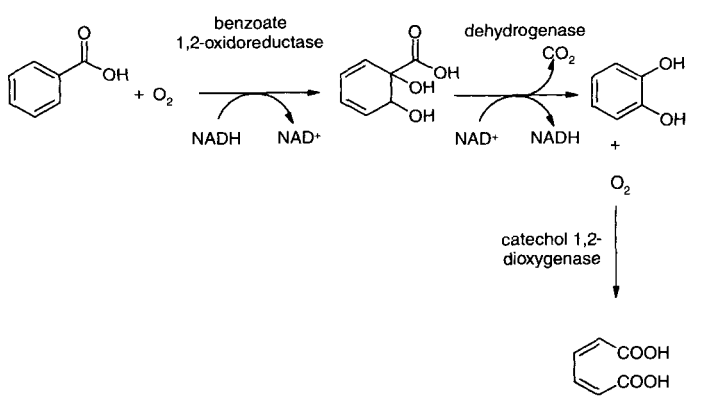

Figure 16.6-6. Sequential oxidation of benzoate to (*cis,cis*)-muconic acid catalyzed by *Arthrobacter* sp.

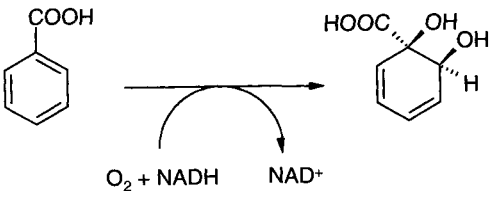

Figure 16.6-7. Dioxygenation of benzoate to corresponding *cis*-1,2-diols.

Benzoic acid was fed continuously to the fermentation medium. The space-time yield of the process including downstream processing amounts to 70 g L^{-1} d^{-1}.

16.6.4.3

Biotransformation of Substituted Benzoates to the Corresponding *cis*-Diols

Enantiopure 1,2-*cis*-dihydroxycyclohexa-3,5-diene carboxylic acids have considerable synthetic potential as building blocks in chiral synthesis. Such *cis*-diols can be produced from benzoic acid derivatives by the action of toluate-1,2-dioxygenase of *Pseudomonas putida* mt-2[25] or homologous enzymes of a different origin (Fig. 16.6-7).

Growing cells or recombinant *Pseudomonas oleovorans* GPo12 containing toluate-1,2-dioxygenase efficiently transform a whole range of *meta*- and *para*-substituted benzoates to the corresponding *cis*-diols, which are not further degraded by the *Pseudomonas* host. In the *ortho* position only hydrogen and fluorine were accepted as substituents. Toluate-1,2-dioxygenase activity is induced by *ortho*-toluate or the substrates themselves.

Similar reactions were reported for the broad-substrate-specific benzoate dioxygenase of *Rhodococcus* sp. strain 19070[26]. Recombinant *E. coli* containing this enzyme transform benzoate and anthranilate to catechol and 2-hydro-1,2-dihydroxybenzoate, respectively.

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16.7
Oxidation of C-N Bonds

Andreas Schmid, Frank Hollmann, and Bruno Bühler

16.7.1
Introduction

Enzymatic oxidations of carbon-nitrogen bonds are as diverse as the substances containing this structural element. Mainly amine and amino acid oxidases are reported for the oxidation of C-N bonds. The steroespecificity of amine-oxidizing enzymes can be exploited to perform resolutions and even deracemizations or stereoinversions (Fig. 16.7-1 A). Analogous to the oxidation of alcohols, primary amines are oxidized to the corresponding imines, which can hydrolyze and react with unreacted amines (Fig. 16.7-1 B). In contrast to ethers, internal C-N bonds are readily oxidized, yielding substituted imines. This can be exploited for the production of substituted pyridines (Fig. 16.7-1 C). Furthermore, pyridines can be oxidized not only to N-oxides but also to α-hydroxylated products (Fig. 16.7-1 D).

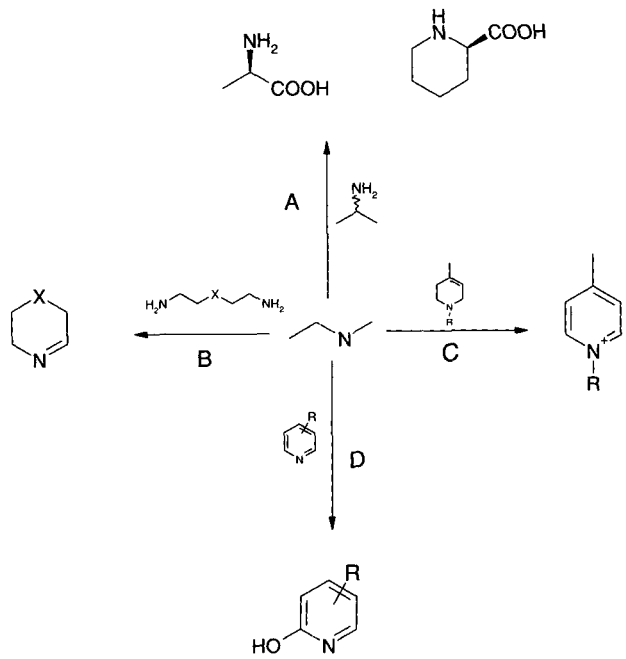


Figure 16.7-1. Oxidations of C-N bonds with synthetic relevance. A: kinetic resolution, deracemization and stereoinversion (Sects. 16.7.2.1 and 16.7.3.1); B: preparation of aldehydes (and subsequent formation of imines) by oxidation of primary amines (Sect. 16.7.3.2); C: preparation of substituted pyridines (Sect. 16.7.3.2); D: hydroxylation of N-heteroaromatic compounds (Sect. 16.7.2.2).

16.7.2
Oxidations Catalyzed by Dehydrogenases

16.7.2.1
L-Alanine Dehydrogenase (L-Ala-DH, E.C. 1.4.1.1)

L-Alanine dehydrogenase (L-Ala-DH, E.C. 1.4.1.1) catalyzes the specific deaminative oxidation of L-alanine and thus can potentially be exploited for the resolution of racemic alanine (e.g. derived from the Strecker-synthesis). However, the oxidation of secondary alcohols and amines is thermodynamically unfavorable[1], so that the equilibrium of the reversible dehydrogenase reaction is on the substrate side. Therefore, an additional thermodynamic driving force has to be introduced into the system in order to drive the desired reaction towards completion. Moiroux and coworkers recently introduced such a system (Fig. 16.7-2)[2–5].

The general philosophy of their approach is the utilization of electrical power to remove the dehydrogenase products NADH and pyruvate (which is *in situ* transformed into the corresponding imine), thus driving the equilibrium reaction towards completion. The electrochemical oxidation and reduction reactions produce NAD^+ and racemic alanine, respectively, as substrates for the dehydrogenase reaction. Using this procedure, not only a racemate resolution (with maximum 50% yield) but a deracemization (100% yield) is achieved. The overall rate-limiting step is the slow, non-enzymatic formation of the imine. Consequently, the process is very slow (at best, the complete conversion of a 10 mM solution of L-alanine required 140 h).

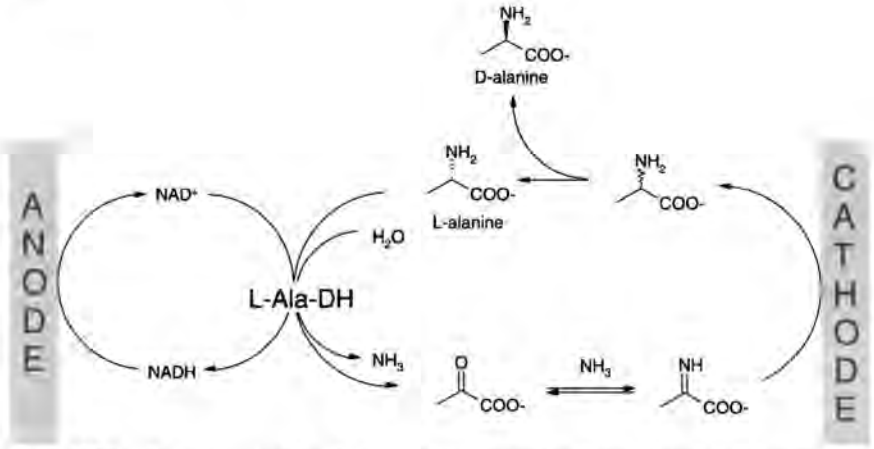


Figure 16.7-2. Stereoinversion of L-alanine to D-alanine catalyzed by L-alanine dehydrogenase (L-Ala-DH) in an electrochemical reactor.

16.7.2.2

Nicotinic Acid Dehydrogenase (Hydroxylase) (E. C. 1.5.1.13)

The membrane-bound molybdoenzyme[6] nicotinic acid dehydrogenase catalyzes the first step in the microbial degradation of nicotinic acid by inserting a hydroxyl function α to the nitrogen atom (Fig. 16.7-3). A possible mechanism for this reaction is given in Fig. 16.7-4[7].

The inserted hydroxyl function originates from water, which was confirmed by $H_2{}^{18}O$ experiments[6, 8]. While nicotinic acid dehydrogenase does not accept NAD^+ as electron acceptor, artificial mediators such as benzyl viologene and 2,3,5-triphenyltetrazolium dyes can replace $NADP^+$[9]. Various bacterial strains have been reported to convert a broad range of nicotinic acid derivatives (Table 16.7-1)[10, 12].

An industrial process (according to the first entry in Table 16.7-1) was set up by

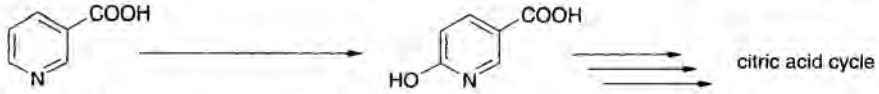


Figure 16.7-3. Microbial mineralization of nicotinic acid.

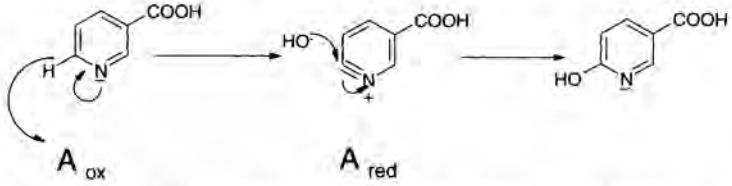
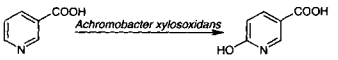
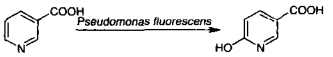
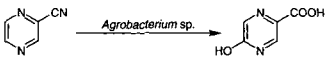
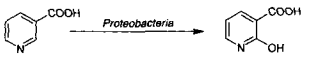
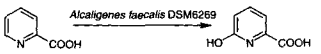
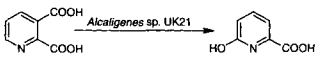
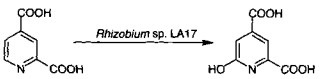
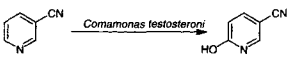
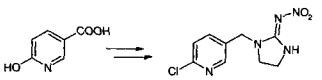
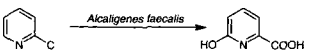
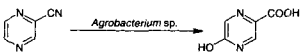
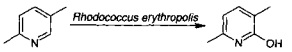


Figure 16.7-4. Proposed mechanism for enzymatic hydroxylation of nicotinic acid (A = acceptor). The reaction scheme is based on the so-called arine mechanism.

Table 16.7-1. Microbial α-hydroxylation of substituted pyridines.

Reactions catalyzed by whole cells	Final product concentration [g L^{-1}]	Enzymes and reference
	74	Dehydrogenase [1]
	191	Dehydrogenase [2]
	301	Dehydrogenase [3]
	6.4	Dehydrogenase [4]
	98	Dehydrogenase [5]
	NR[a]	Dehydrogenase and decarboxylase [6]
	NR[a]	Dehydrogenase [7]
	45	Dehydrogenase [8]
	40	Nitrilase and Dehydrogenase [9]
	55	Nitrilase and Dehydrogenase [5]
	40	Nitrilase and Dehydrogenase [10]
	8	Dehydrogenase [11]

a NR: not reported.

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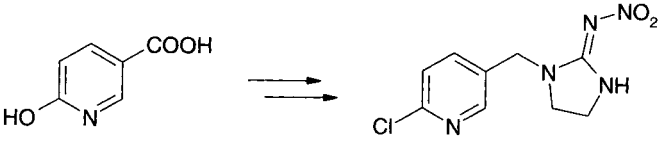


Figure 16.7-5. 6-Hydroxynicotinic acid as synthon for the pesticide Imidachloprid.

Lonza AG, Switzerland. 6-Hydroxynicotinic acid is precipitated from the fermentation broth as magnesium salt in the so-called pseudocrystal process, thus enabling not only easy downstream processing but also continuous fermentation[13]. 6-Hydroxynicotinic acid is the key building block in the synthesis of Imidachloprid (Fig. 16.7-5), an effective pesticide against hemipterans and other sucking insects[10, 11].

16.7.3
Oxidations Catalyzed by Oxidases

16.7.3.1
Amino Acid Oxidases

Among the enzymes catalyzing oxidations of carbon nitrogen bonds, the amino acid oxidases (AAO, E.C. 1.4.3.x) are the most interesting for synthetic applications. Compared to some specific amino acid oxidases such as aspartate oxidase or glutamate oxidase, the two D- and L-amino acid oxidases (E.C. 1.4.3.2 for L-AAO and E.C. 1.4.3.3 for D-AAO) are advantageous on account of their broad substrate

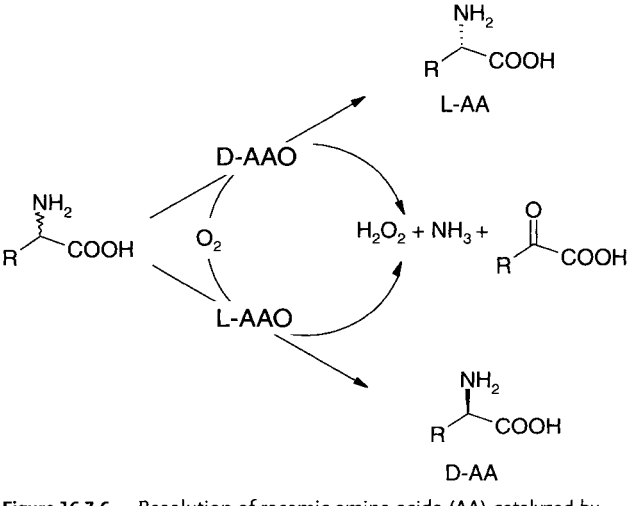


Figure 16.7-6. Resolution of racemic amino acids (AA) catalyzed by (D)- and (L)-specific amino acid oxidases (AAO).

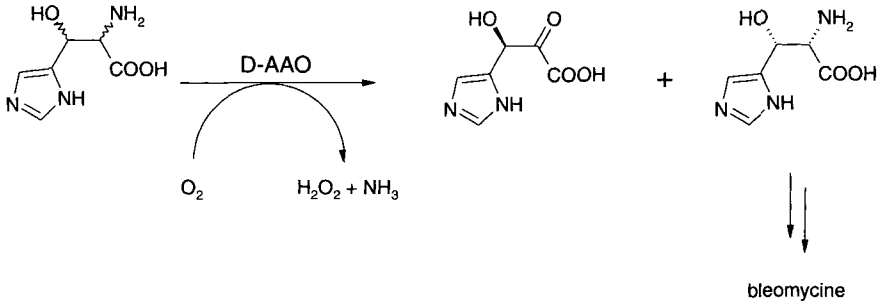


Figure 16.7-7. Resolution of D,L-*erythro*-β-hydroxyhistidine as the enantiospecific step in bleomycine synthesis.

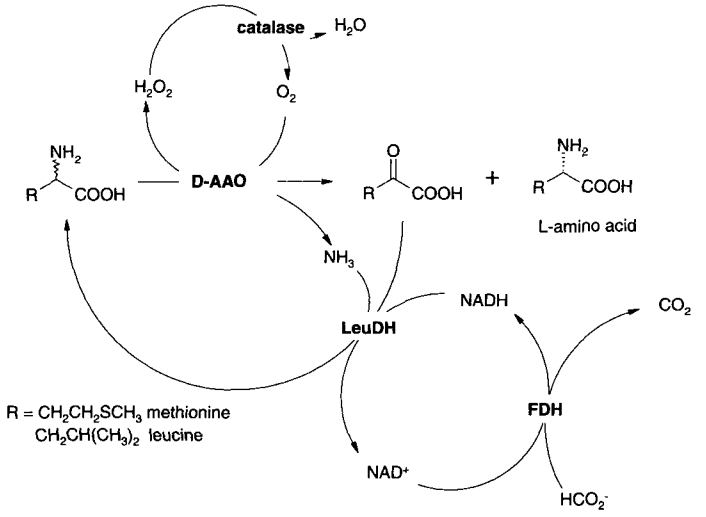


Figure 16.7-8. Enzymatic deracemization of amino acids catalyzed by D-amino acid oxidase (D-AAO). Leucine dehydrogenase (LeuDH) transforms the oxidation product of the undesired amino acid enantiomer *in situ* into the racemic amino acid. Regeneration of NADH is performed by formate dehydrogenase (FHD).

spectrum and their strict stereospecificity[14, 15]. Therefore, AAOs are most commonly used for the resolution or deracemization of racemic amino acid mixtures (Fig. 16.7-6).

The approach outlined in Fig. 16.7-6 was used for example to remove traces of D-methionine from 99% pure L-methionine[16, 17] or to transform racemic phenylalanine quantitatively into D-phenylalanine and phenylpyruvic acid[18]. Coimmobilization with catalase on a solid matrix (Eupergit®) resulted in largely increased D-AAO stability. In an enzyme-membrane-reactor, space-time-yields as high as 90 g L^{-1} d^{-1} were reached. In another example, a racemic mixture of D,L-erythro-β-hydroxyhistidine was converted into the ketoacid and L-erythro-β-hydroxyhistidine[19]. The

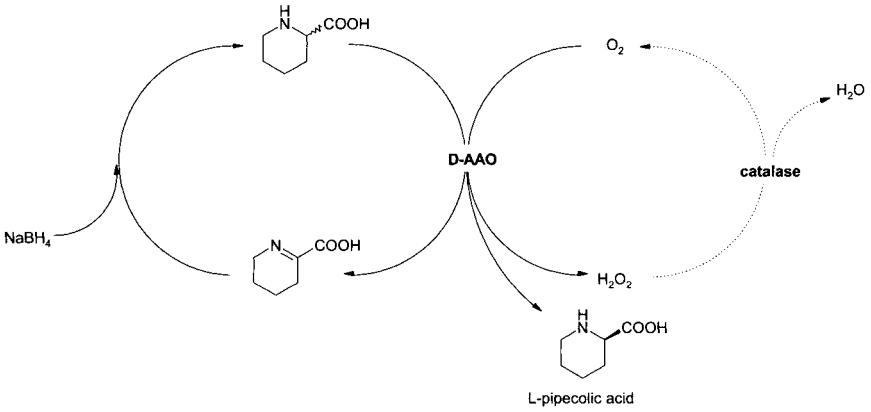


Figure 16.7-9. One-pot chemo-enzymatic deracemisation of D,L-pipecolic acid catalyzed by D-amino acid oxidase (D-AAO). Utilization of catalase was not reported.

latter compound is a key intermediate in the synthesis of the anti-tumor agent bleomycine (Fig. 16.7-7)

Simple racemate resolutions have a maximal yield of 50% for the desired compound. Furthermore, additional (potentially laborious) separation steps are necessary. As a consequence, alternative processes that involve the stereoinversion of the undesired enantiomer are gaining increasing interest[20]. One approach for these so-called deracemization processes is to reconvert the oxidation product either enzymatically (Fig. 16.7-8) or chemically (Fig. 16.7-9) to the racemic substrate.

The enzymatic variant of this concept was reported for the deracemization of D,L-methionine or D,L-leucine (Fig. 16.7-8)[17]. Soda and coworkers developed a chemo-enzymatic racemization procedure utilizing boron hydrides for non-enantioselective reduction of the undesired D-AAO product (Fig. 16.7-9)[21, 22]. Using the same procedure, the authors achieved conversion of D-proline into L-proline[21]. Furthermore, D,L-lactate and 2-hydroxy butyric acid were deracemized by utilizing L-lactate oxidase[23].

The D-AAO catalyzed oxidative deamination of cephalosporin C found industrial application (Hoechst Marion Roussel, Germany) as the first step in the so-called 7-aminocephalosporanic acid (7-ACA) process (Fig. 16.7-10)[24–26].

Using this process, this application of heavy metals and chlorinated hydrocarbons can be avoided, and the volumes of waste-gas as well as of mother liquors are drastically reduced[26].

16.7.3.2
Amine Oxidases

16.7.3.2.1 Monoamine Oxidase (MAO, E.C. 1.4.3.4)

The flavoenzymes monoamine oxidase A and B (MAO-A, MAO-B)[27] catalyze the oxidative deamination of various primary and secondary amines and the oxidation of tertiary amines. Their physiological role, as the various synonyms such as epineph-

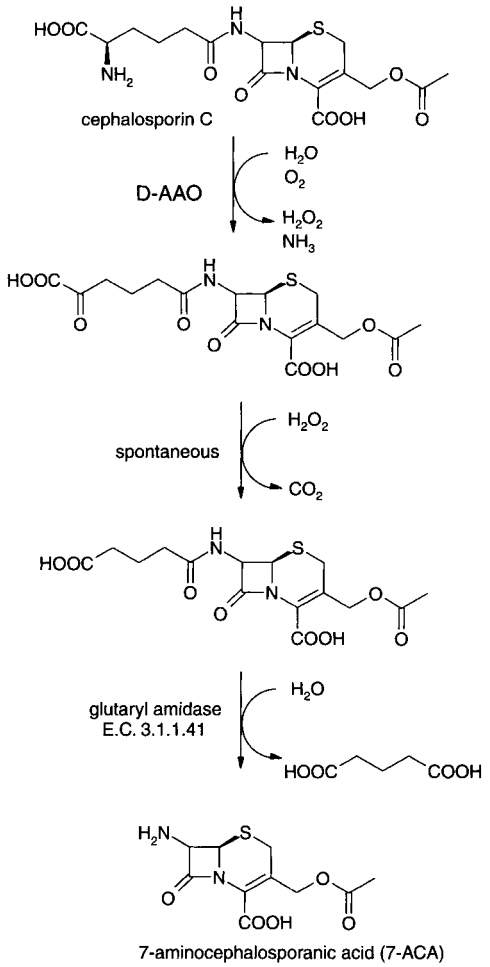


Figure 16.7-10. Enzymatic reaction sequence for the production of 7-ACA from cephalosporin C.

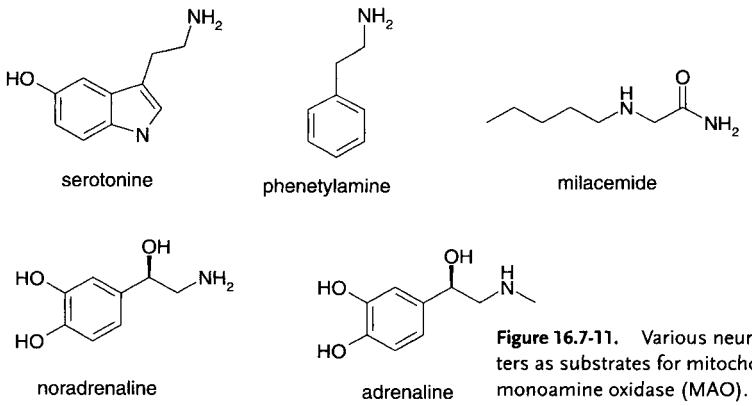


Figure 16.7-11. Various neurotransmitters as substrates for mitochondrial monoamine oxidase (MAO).

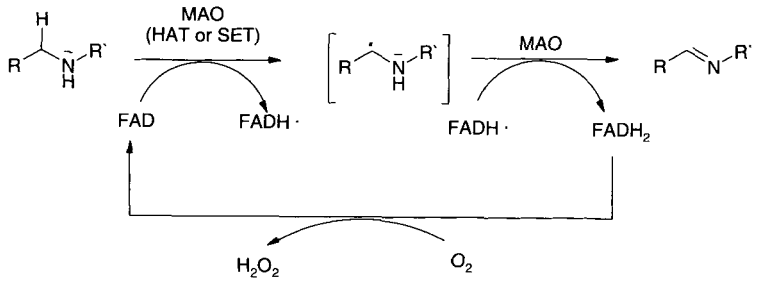


Figure 16.7-12. Proposed mechanisms for the oxidation of primary and secondary amines by monoamine oxidase (MAO).

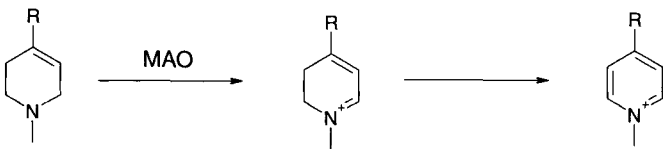


Figure 16.7-13. Oxidation of 1-methyl-4-aryl(heteroaryl)-1,2,3,6-tetrahydropyridines catalyzed by monoamine oxidase (MAO).

rine oxidase, serotonin oxidase, tyramine oxidase, or adrenaline oxidase suggest, is the transformation of neurotransmitters via oxidative deamination (Fig. 16.7-11) as well as the detoxification of xenobiotics[28–30].

The mechanism of MAO is still a topic of debate[31]; hydrogen atom transfer (HAT)[32] or single electron transfer (SET)[33] are discussed as initial oxidation steps in the overall mechanism (Fig. 16.7-12).

Various substrates have been specified for MAO with respect to synthetical, mechanistical and biochemical purposes. Castagnoli and coworkers elucidated structural requirements of MAO-B with various substituted 1-methyl-1,2,3,6-tetra-

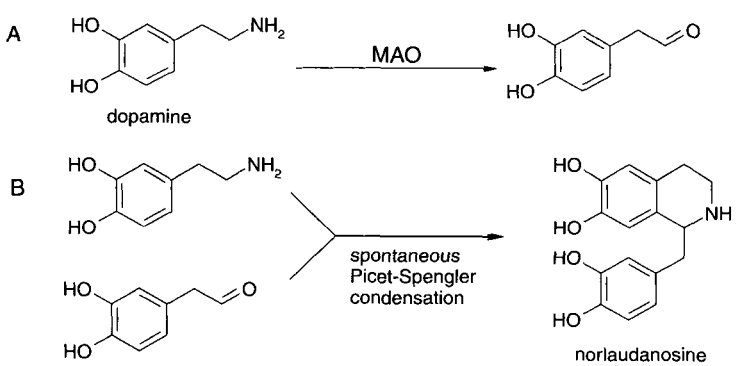
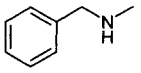
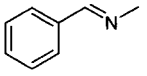
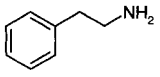
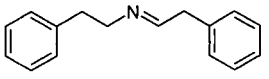
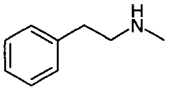
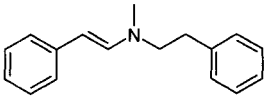
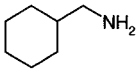
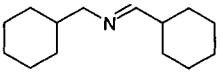
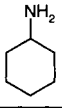
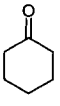


Figure 16.7-14. Preparation of norlaudanosine initiated by the oxidation of dopamine by monoamine oxidase (MAO) (A). The oxidation product reacts spontaneously in a Picet-Spengler condensation with unreacted dopamine (B).

Table 16.7-2. Oxidation of various amines catalyzed by monoamine oxidase in *n*-octane (0.5 % v/v water) [12].

Substrate	Product	Yield [%]
		99
		99
		92
		69
		14

12 J. C. G. Woo, X. Wang, R. B. Silverman, *J. Org. Chem.* **1995**, *60*, 6235–6236.

hydropyridines to produce dihydropyridines that are further oxidized to pyridinium structures (Fig. 16.7-13) [31, 34].

MAO was used *in vivo* and *in vitro* as a catalyst for the production of norlaudanosine from dopamine (Fig. 16.7-14) [35]. Norlaudanosine is an important synthon for benzylisoquinoline alkaloids, providing the upper isoquinoline portion of the morphinan skeleton. *In vitro* and *in vivo* yields were in the range of 20 %.

MAO-B was also tested in low water content organic media such as ether, tetrachloromethane, octane, benzene and cyclohexane. Under optimized conditions quantitative conversions of various substrates were achieved (Table 16.7-2) [36].

16.7.3.2.2 **Diamine Oxidase (E. C. 1.4.3.6)**

The copper-containing amine oxidases (copper amine oxidases, diamine oxidases) possess either a topaquinone or a 6-hydroxydopamine cofactor (Fig. 16.7-15), generally integrated in the oxidase primary structure. Tyrosine residues of the enzyme backbone in the active site are discussed as precursors for the prosthetic group [37].

As the name suggests, diamine oxidase catalyzes the oxidative deamination of diamines. Preferably α,ω-diamines such as putrescine (1,4-diaminobutane) or cadaverine (1,5-diaminopentane) (the names already suggest their smell), but also various derivatives are readily converted. Quite often cyclic imines are obtained via internal nucleophilic attack by the unreacted amino function (Fig. 16.7-16) [38–40].

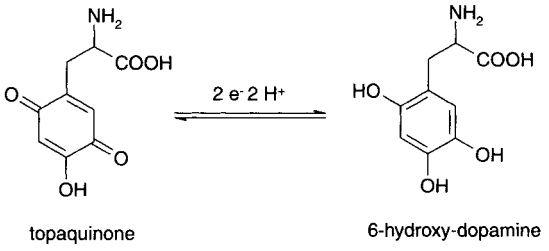


Figure 16.7-15. Topaquinone and 6-hydroxy-dopamine as prosthetic groups of diamine oxidases.

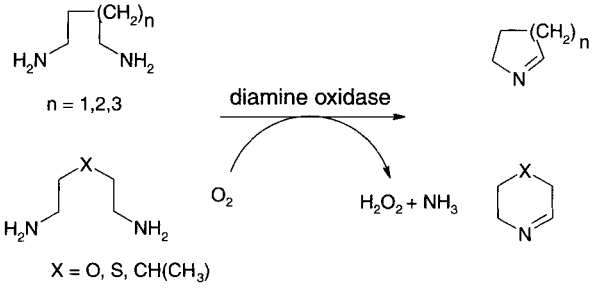


Figure 16.7-16. Application of diamine oxidase in the synthesis of different azaheterocycles.

In the presence of suitable nucleophiles (such as benzoyl acetic acid) the primary imines can be spontaneously further modified *in situ*. A convenient approach to obtain phenacyl-derivatives, building blocks in the synthesis of certain alkaloids, was reported[38]. In some cases, diamine oxidases exhibit activities complementary to monoamine oxidases. For example vanillylamine is far more efficiently converted into vanillin by a diamine oxidase from *Aspergillus niger* than by the monoamine oxidase from *E. coli*[11].

Even enantioselective oxidations of some alkyl-, benzyl-, or phenylethyl- (arylethyl-) amines were reported with diamine oxidase from pea settlings[41]. Porcine kidney diamine oxidase was used for the oxidative transformation of *Nitraria* alkaloids such as nazlinin[42].

For the conversion of poorly water-soluble amines (and to avoid product inhibition), diamine oxidase can also be applied in non-aqueous media[43].

16.7.4
Oxidations Catalyzed by Transaminases

Transaminases are generally not considered to be enzymes catalyzing redox reactions, which is obvious considering the meaning of the E.C. code for transferases (E.C. 2.6.1.x = transferring amino groups). Nevertheless, the exchange of an amino functionality between an amino acid and an α-keto acid implies the oxidation of the amino acid. Transaminases are described elsewhere in this book (Chapter 12).

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Karlheinz Drauz and Herbert Waldmann
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16.8
Oxidation at Sulfur

Karl-Heinz van Pee

16.8.1
Enzymes Oxidizing at Sulfur and their Sources

The oxidation at sulfur is catalyzed by a number of different enzymes produced by a variety of organisms. They have been isolated from a fungus[1], soybean[2], rat, pig and rabbit liver[3–5], horseradish[6], bacteria[7–9], milk[10], and human white blood cells[11].

The enzymes catalyzing oxidation reactions at sulfur belong to two different classes of enzymes: monooxygenases, including cytochrome P-450 monooxygenases and FAD-containing monooxygenases, and heme-containing peroxidases (Figs. 16.8-1 and 16.8-2, Table 16.8-1).

Some of these enzymes such as chloroperoxidase from *Caldariomyces fumago*, horseradish peroxidase, lactoperoxidase from bovine milk, and myeloperoxidase from human white blood cells are commercially available.

Others such as pig liver microsomal FAD-containing monooxygenase have to be isolated from tissue with very low yields[4] or like hydrocarbon monooxygenase from *Pseudomonas oleovorans*[12–13] require several protein components and cofactors, substantially limiting the use of these enzymes for the production of oxidized sulfur compounds.

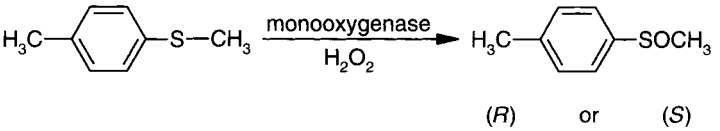

Figure 16.8-1. Oxidation of methyl *p*-tolyl sulfide to methyl *p*-tolyl sulfoxide by a monooxygenase. The product can either be of the *R*- or *S*-configuration depending on the monooxygenase used.

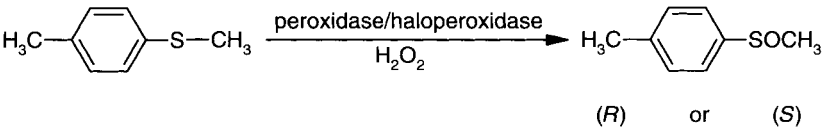

Figure 16.8-2. Oxidation of methyl *p*-tolyl sulfide to methyl *p*-tolyl sulfoxide by a peroxidase or haloperoxidase in the presence of hydrogen peroxide. The product can either be predominantly of the *R*- or *S*-configuration depending on the peroxidase or haloperoxidase used.

Table 16.8-1. Classification of enzymes oxidizing at sulfur and their sources.

Enzyme class	Source	Reference
Monooxygenases	pig liver microsomes	14
	rat liver microsomes	3
	rabbit liver microsomes	16
	bovine adrenals	17
	Pseudomonas oleovorans	12, 13
	Acinetobacter sp.	15
Peroxidases	soybean	2
	horseradish	23
Haloperoxidases	*Caldariomyces fumago*	1
	Ascophyllum nodosum	27
	Corallina officinalis	28
	human white blood cells	30
	bovine milk	25

16.8.2
Oxidation of Sulfides

16.8.2.1
Oxidation of Sulfides by Monooxygenases and by Whole Organsims

Fujimori et al.[14] used pig liver microsomal FAD-containing monooxygenase and phenobarbital-induced rabbit liver microsomal cytochrome P-450 to catalyze the oxidation of unsymmetrical sulfides to the corresponding optically active sulfoxides with varying degrees of enantiomeric excess (12–96%). Comparison of the oxygenation of racemic 2-methyl-2,3-dihydrobenzo[*b*] thiophene showed that the enantiotopic, diastereotopic, and enantiomeric differentiating abilities of the FAD-containing monooxygenase are higher than those of the cytochrome P-450 monooxygenase. They found that the oxygenation with the FAD-containing monooxygenase is sterically much more highly controlled than that with cytochrome P-450. Whereas higher ee-values are observed in the oxygenation of smaller sulfides with the FAD-containing monooxygenase, the oxygenation of large sulfides by the cytochrome P-450 monooxygenase results in higher ee values than those of sulfides bearing small substituents.

Hydrocarbon monooxygenase from *Pseudomonas oleovorans*[7, 8, 12] also catalyzes the stereoselective sulfoxidation of methyl thioether substrates[13] with up to 80% ee. The products obtained with this enzyme are probably of the *R*-configiration.

The (*S*)-(–)-sulfoxide is predominantly produced (82% *S*, 18% *R*) from *p*-tolyl ethyl sulfide when cyclohexanone monooxygenase from *Acinetobacter* sp. NCIB 9871[9] was used, whereas the the FAD-containing monooxygenase from hog liver microsomes oxidizes *p*-tolyl ethyl sulfide to yield the (*R*)-(+)-sulfoxide enantiomer as the major product (95% *R*, 5% *S*)[15].

The enzymatic oxidation of various diaryl, dialkyl, and aryl alkyl sulfides by cytochrome P-450 from rabbit liver resulted predominantly in the formation of the sulfoxides with the *R*-configuration[16].

The *S*-(-) configuration was predominantly obtained when two cytochrome P-450 isoenzymes from rat liver were used for the oxidation of *p*-tolyl ethyl sulfides[3].

Oxidation of phenyl 2-aminoethyl sulfide by dopamine β-hydroxylase from bovine adrenals in the presence of ascorbate as the electron donor resulted in the formation of phenyl 2-aminoethyl sulfoxide. The product was probably of the *S*-configuration[17].

Holland et al.[18] obtained the (*R*)-sulfoxides from various *para*-substituted phenyl 3-chloropropyl and phenyl 3-hydroxypropyl sulfides by biotransformation with the fungus *Mortierella isabellina* with an enantiomeric excess of 82–88%. The (*S*)-sulfoxides were produced using the fungus *Helminthosporium* sp. and the bacterium *Acinetobacter calcoaceticus* with ee values of > 95% and 94%, respectively.

16.8.2.2
Oxidation of Sulfides by Peroxidases and Haloperoxidases

A number of peroxidases were investigated for their use in oxidizing organic sulfides. *p*-Substituted thioanisols were oxidized by partially purified soybean sulfoxidase using 13(*S*)-hydroperoxylinoleic acid as the peroxide. Methyl *p*-tolyl sulfide gave the (*S*)-sulfoxide with about 90% ee[2].

The sulfoxidation of organic sulfides by chloroperoxidase from *Caldariomyces fumago* was investigated by different groups[19–26]. Colonna et al.[20] compared the oxidation of sulfides by this enzymes with that catalyzed by horseradish peroxidase. Chloroperoxidase catalyzed the formation of sulfoxides with *tert*-butyl and other peroxides with an *R* absolute configuration in up to 92% ee, whereas horseradish peroxidase gave racemic products. When sterically hindered oxidants such as cumyl hydroperoxides and chloroperoxidase were used, racemic or almost racemic products were obtained. *tert*-Butyl hydroperoxide also had the advantage of giving higher yields and higher ee.

Using vanadium bromoperoxidases from marine algae the (*S*)- or (*R*)-sulfoxides can be obtained from methyl phenyl sulfide derivatives, respectively, depending on the source of the enzyme. While bromoperoxidase from *Ascophyllum nodosum* produces the (*R*)-sulfoxide with 91% ee[27], the (*S*)-enantiomer is obtained with bromoperoxidases from *Corallina officinalis* and *C. pilulifera*[28].

When investigating the substrate selectivity using a series of aryl, alkyl, dialkyl, and heterocyclic sulfides, it was found that *p*-substitution led to higher enatioselectivity and higher chemical yields with respect to *o*-substitution[20]. A similar influence of the *p*-substitution was found for sulfoxidation catalyzed by bromoperoxidase from the marine alga *Ascophyllum nodosum*[27].

Benzyl methylsulfide, thioanisol, and thiobenzamide were oxidized by chloroperoxidase, lactoperoxidase, and horseradish peroxidase to the respective sulfoxides. Whereas lactoperoxidase and horseradish peroxidase had low activities towards benzyl methylsulfide, thiobenzamide was efficiently oxidized by lactoperoxidase. Chloroperoxidase had high activity in halide-independent reactions towards all three substrates[25]. This enzyme was also used for the asymmetric sulfoxidation of a series of cyclic sulfides. In all cases the (*R*)-sulfoxides were obtained. In the case of

Table 16.8-2. Products and absolute configuration obtained in the oxidation of various sulfides by different enzymes.

Sulfide	Predominant configuration of sulfoxide obtained	Enzyme or organism	Reference
Methyl phenyl	*R*	chloroperoxidase	19–21
	R	vanadium bromoperoxidase	27
	S	vanadium bromoperoxidase	28
Methyl *p*-tolyl	*S*	soybean hydroperoxide-dependent oxygenase	2
Ethyl *p*-tolyl	*S*	rat liver cytochrome P-450	3
		cyclohexanone monooxygenase	15
	R	FAD-containing monooxygenase	15
Methyl alkyl	*R*	alkane monooxygenase	13
Diaryl, dialkyl, aryl alkyl	*R*	rabbit liver cytochrome P-450	16
Phenyl 2-aminoethyl	*S*	dopamine β-hydroxylase	17
Phenyl 3-chloropropyl	*R*	*Mortierella isabellina*	18
Phenyl 3-chloropropyl	*S*	*Helminthosporium* sp.	18
2,3-Dihydrobenzo[*b*]thiophene	*R*	chloroperoxidase	29

2,3-dihydrobenzo[*b*]thiophene the yield was 99.5% with an ee of 99%[29]. Table 16.8-2 shows some examples of sulfides oxidized to sulfoxides by different enzymes and the absolute configuration of the products. When using peroxidases, care has to be taken, as the peroxidase-catalyzed oxidation is in competition with the spontaneous oxidation of the sulfides by the oxidant.

Depending on the enzyme used for oxidation of organic sulfides, sulfoxides with *S*- or *R*-configuration can be obtained with high ee, whereas at present there is only one chemical oxidation method which leads to high ee in alkyl aryl sulfoxides. This method uses chiral titanium complexes and cumene hydroperoxide for the oxidation of organic sulfides[26].

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16.9 Halogenation

Karl-Heinz van Pee

16.9.1 Classification of Halogenating Enzymes and their Reaction Mechanisms

16.9.1.1 Haloperoxidases and Perhydrolases

The only type of halogenating enzymes known until 1997 were peroxidases and perhydrolases which catalyze the formation of carbon halogen bonds using halide ions, hydrogen peroxide and an organic substrate activated for electrophilic attack.

According to the halide ions they can utilize they are arranged into three groups: iodoperoxidases, bromoperoxidases and chloroperoxidases. Iodoperoxidases catalyze the formation of carbon-iodine bonds, whereas bromoperoxidases catalyze iodination and bromination reactions, and chloroperoxidases catalyze the iodination, bromination, and chlorination of organic substrates. As haloperoxidases are oxidoreductases using hydrogen peroxidase as the oxidant for the oxidation of halide ions producing hypohalogenic acids, the existence of fluoroperoxidases can be ruled out. The overall reactions catalyzed by haloperoxidases and perhydrolases are shown in Fig. 16.9-1. All haloperoxidases isolated until 1984 were heme-containing enzymes[1]. The first non-heme haloperoxidase was isolated by Vilter[2]. Instead of heme, vanadium is responsible for the halogenating activity of this algal enzyme[3, 4]. Non-heme and non-metal "haloperoxidases" were isolated from bacteria[5–9], however, elucidation of the three-dimensional structure and the reaction

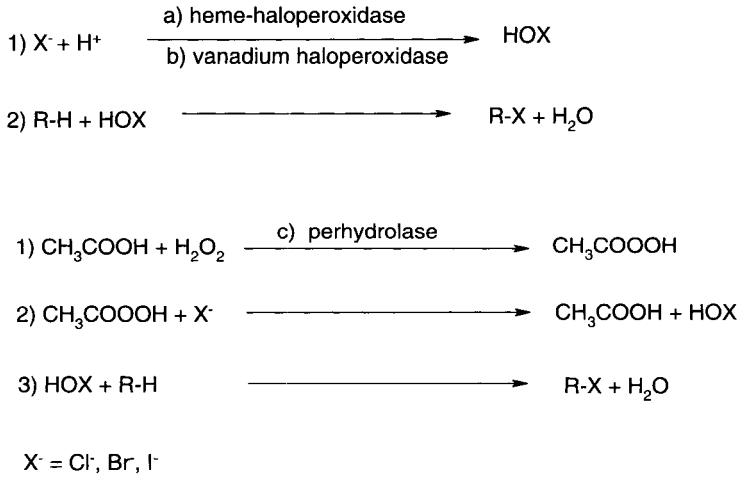


Figure 16.9-1. Overall reaction catalyzed by (a) heme-[51] and (b) vanadium-containing haloperoxidases[51] and (c) perhydrolases[20].

mechansim of this type of halogenase showed that they are not real haloperoxidases. They are actually perhydrolases which produce hypohalogenic acids via the oxidation of halide ions by enzymatically formed peracetic acid[10–12]. Thus, in addition to grouping the haloperoxidases according to the range of halide ions oxidized, they can be classified according to their prosthetic group into heme type and non-heme type haloperoxidases[13].

The heme type haloperoxidases are inactivated during the halogenation reaction, because the heme group of these enzymes is attacked by the hypohalous acids produced by the enzymes[1]. Thus, heme type haloperoxidases have the disadvantage that the reaction velocity slows down considerably during the course of the reaction[14]. With non-heme type haloperoxidases this does not seem to be the case. They are not inactivated during the halogenation reaction and are very stable under reaction conditions[14]. However, the disadvantage of inactivation is partly compensated for by the fact that some of the heme type haloperoxidases have much higher specific activities than non-heme type haloperoxidases.

Some of the non-heme haloperoxidases are very stable with respect to organic solvents[15] which is of great importance when the substrates that are to be halogenated are not very soluble in water. In these cases water missible organic solvents can be added to the reaction mixture or a two phase-system can be used.

16.9.1.2
FADH$_2$-dependent Halogenases

In 1997 the existence of a novel class of halogenating enzymes was reported[16]. These halogenases showed no relationship to any of the known haloperoxidases[17, 18] and did not require hydrogen peroxide for halogenating activity. Initially these new halogenases were thought to require NADH[16], but more detailed studies showed that they actually require FADH$_2$[19, 20] which is produced by NADH-dependent flavin reductases. Figure 16.9-2 shows the hypothetical reaction mechanism of FADH$_2$-dependent halogenases.

16.9.2
Sources and Production of Enzymes

16.9.2.1
FADH$_2$-dependent Halogenases

Although FADH$_2$-dependent halogenases seem to be present in many bacteria producing halometabolites[19, 21, 22], only one example of this new class of halogenases has been isolated to homogeneity until now. This enzyme, tryptophan 7-halogenase, is produced by several *Pseudomonas* strains producing the antibiotic pyrrolnitrin such as *Pseudomonas fluorescens* and *P. aureofaciens* and by *Myxococcus fulvus*[23]. Monodechloroaminopyrrolnitrin-3-halogenase, another FADH$_2$-dependent halogenase from pyrrolnitrin-producing *Pseudomonas* strains has so far only been purified partially[16, 20].

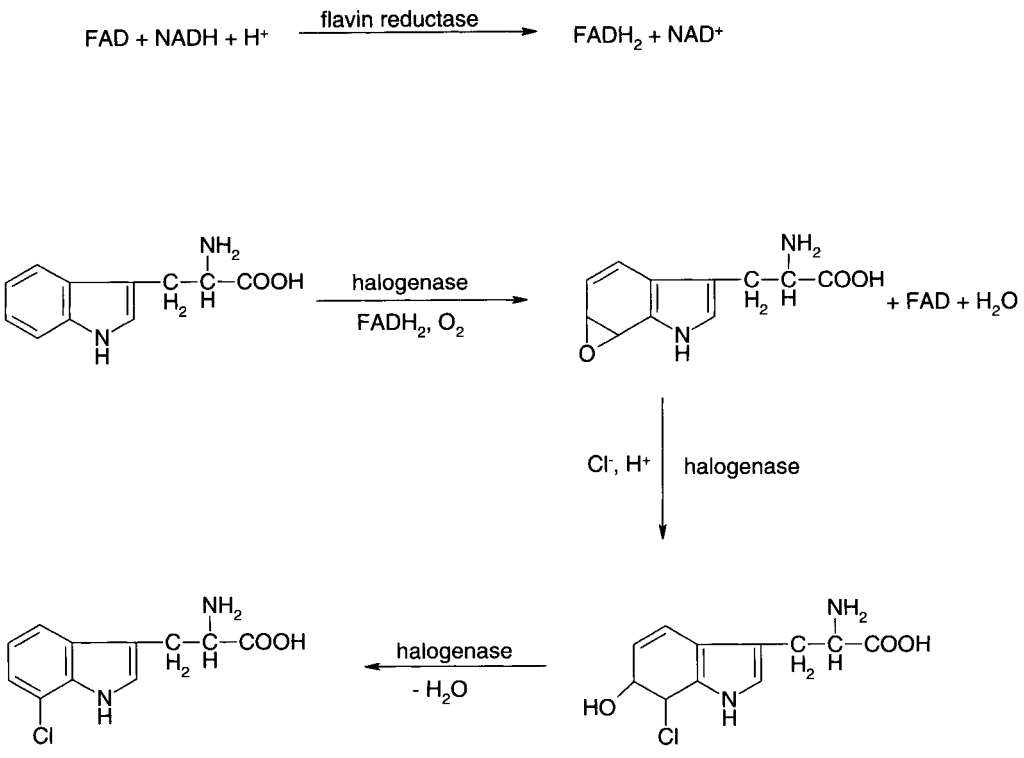


Figure 16.9-2. Hypothetical reaction mechanism of $FADH_2$-dependent tryptophan 7-halogenase as an example of $FADH_2$-dependent halogenases[20].

From biosynthetic and hybridization studies it is known that $FADH_2$-dependent halogenases are involved in the biosynthesis of many halometabolites produced by bacteria[19, 21, 22] and it can be expected that other $FADH_2$-dependent halogenases will be purified and characterized in the near future.

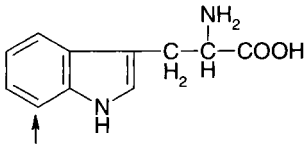
16.9.2.2
Haloperoxidases and Perhydrolases

Iodoperoxidases such as horseradish peroxidase[24] and thyroid peroxidase[25] can be isolated from many different organisms. Bromoperoxidases have been obtained in a pure form from mammals (lactoperoxidase)[26], sea urchin (ovoperoxidase)[27], marine algae[28–30], lichen[31], fungi (lignin peroxidase)[32] and bacteria[33, 34]. Chloroperoxidases have been found in mammals (myeloperoxidase[35] and eosinophil peroxidase[36]), a marine worm[37], and fungi[38–40]. Several perhydrolases have been isolated from bacteria[5–9, 41].

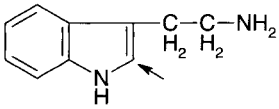
Chloroperoxidase can be produced in batch culture at concentrations of 280 mg L^{-1}[42] and 20 mg of lactoperoxidase can be isolated from 1 L of bovine milk[26]. The sources for these two enzymes, bovine milk and culture broth of *Caldariomyces*

fumago, are easily obtained. Chloroperoxidase can also be obtained in larger quantities from the fungus *Curvularia inaequalis*[40]. Thus, a number of different haloperoxidases from various sources are available in quantities necessary for the enzymatic halogenation of organic compounds.

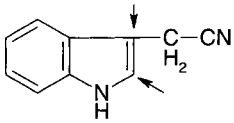
a)



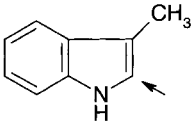
tryptophan



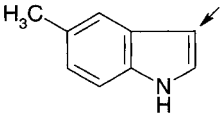
tryptamine



indole-3-acetonitrile

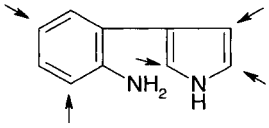


3-methylindole

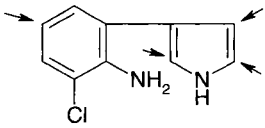


5-methylindole

b)



monodechloroamino-pyrrolnitrin



aminopyrrolnitrin

Figure 16.9-3. Substrates accepted by tryptophan 7-halogenase: (a) indole derivatives, (b) phenylpyrrole derivatives; the positions of chlorination are indicated by arrows[43].

16.9.3
Substrates for Halogenating Enzymes and Reaction Products

16.9.3.1
Halogenation of Aromatic Compounds

The recently detected $FADH_2$-dependent halogenases are substrate specific. Tryptophan 7-halogenase catalyzes the chlorination and bromination of D- and L-tryptophan to 7-chloro- or 7-bromotryptophan, respectively[20]. This enzyme also accepts a number of other indole derivatives such as tryptamine, indole-3-acetonitrile, 3-methylindole and 5-methylindole as substrates (Fig. 16.9-3a)[43]. In addition to indoles, aminophenylpyrrole derivatives are also chlorinated by tryptophan 7-halogenase (Fig. 16.9-3b)[43].

Monodechloroaminopyrrolnitrin 3-halogenase catalyzes the regioselective chlorination of the aminophenylpyrrole derivative monodechloroaminopyrrolnitrin to aminopyrrolnitrin[16], however, nothing is known about the substrate specificity of this enzyme.

In contrast to $FADH_2$-dependent halogenases, haloperoxidases have no substrate specificity. The enzymatic iodination, bromination, and chlorination of a number of different aromatic compounds by haloperoxidases have been reported in the last few years. All aromatic substrates halogenated successfully by haloperoxidases are aromatic compounds activated for electrophilic substitution (Table 16.9-1).

Phenols and phenol ethers are very good substrates for haloperoxidases. The first aromatic substrate to be used in enzymatic iodination was tyrosine. This substrate was iodinated using chloroperoxidase from *Caldariomyces fumago*[44] and thyroid peroxidase[45]. Horseradish peroxidase and lactoperoxidase have been used to lable proteins with radioactive isotopes of iodide[1] and bromoperoxidase from *Penicillus capitatus* has been employed to lable human serum albumin with the radioactive isotope of bromine[46].

Phenolsulfonephthalein (Phenol Red) is brominated to 3,3',5,5'-tetrabromophenolsulfonephthalein (Bromophenol Blue) by many haloperoxidases[1, 15]. This reaction has been used for the detection of halogenating enzymes by different groups[9, 47].

Corbett et al.[48] obtained 2,6-dibromo-4-chloroaniline or 2,4,6-trichloroaniline when they incubated 4-chloroaniline with chloroperoxidase in the presence of hydrogen peroxide and bromide or chloride, respectively.

Several obscurolides, secondary metabolites produced by *Streptomyces viridochromogenes* T7[49], were brominated using perhydrolase from *Streptomyces aureofaciens* Tü24[50]. The obscurolides were monobrominated in the 2-position and dibrominated in the 2,4-positions of the aromatic ring system of the obscurolides (Fig. 16.9-4). In the case of dibromination, the hydroxymethyl group was replaced by bromine. No bromination of the olefinic double bond could be detected.

A number of aromatic heterocyclic compounds have been halogenated by different haloperoxidases.

Franssen et al.[51] used chloroperoxidase from *Caldariomyces fumago* to chlorinate

Table 16.9-1. $FADH_2$-dependent halogenases, haloperoxidases and perhydrolases used for biotransformation of aromatic compounds and their sources.

Enzyme (type)	Source	Substrate (halide)	Reference
Tryptophan 7-halogenase	pyrrolnitrin-producing	indole derivatives (Cl^-, Br^-)	20, 43
($FADH_2$-dependent)	*Pseudomonads*	phenylpyrroles (Cl^-, Br^-)	43
Monodechloroaminopyrrolnitrin 3-	pyrrolnitrin-producing	monodechloroamino-	
halogenase ($FADH_2$-dependent)	*Pseudomonads*	pyrrolnitrin (Cl^-, Br^-)	20
Chloroperoxidase (heme)	*Caldariomyces fumago*	phenol ether (Cl^-, Br^-)	67–68
		phenols (Cl^-, Br^-, I^-)	44
		anilines (Cl^-, Br^-)	48
		pyrazoles, pyridines (Cl^-)	51
		nucleic bases (Cl^-, Br^-, I^-)	14
Lactoperoxidase (heme)	bovine milk	phenols (I^-)	72
		estrone (I^-)	73
Thyroid peroxidase (heme)	thyroid glands	phenols (I^-)	45
Chloroperoxidase (heme-flavin)	*Notomastus lobatus*	phenols (Cl^-, Br^-)	37
Bromoperoxidase (non-heme)	*Asophyllum nodosum*	phenols (Br^-, I^-)	74
		phenol red (Br^-)	15
Bromoperoxidase (non-heme)	*Corallina pilulifera*	nucleic bases (Br^-, I^-)	14
		phenols (Br^-)	75
Perhydrolase (non-heme, non-metal)	*Streptomyces aureofaciens*	nikkomycin (Br^-)	52
		phenylpyrroles (Cl^-, Br^-)	56
		obscurolide (Br^-)	50
		phenol red (Br^-)	9
Perhydrolase (non-heme, non-metal)	*Pseudomonas pyrrocinia*	indole (Cl^-, Br^-)	29
		phenylpyrroles (Cl^-, Br^-)	7, 54, 56

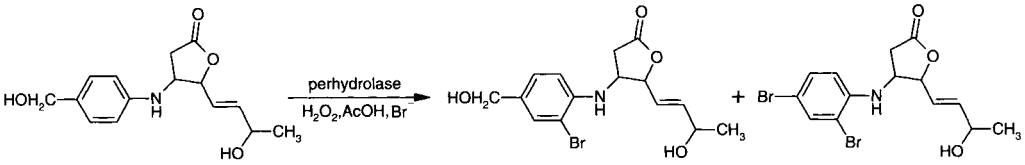


Figure 16.9-4. Bromination of obscurolide A_3 by perhydrolase from *Streptomyces aureofaciens* Tü24[50].

pyrazole, 1-methylpyrazole and 3-methylpyrazole to their corresponding 4-chloroderivatives. The same enzyme was used to produce 5,7-dibromo-8-hydroxyquinoline from 8-hydroxyquinoline. 2-Aminopyridine was regiospecifically chlorinated to 2-amino-3-chloropyridine.

The chlorination, bromination, and iodination of various nitrogen-containing heterocycles catalyzed by chloroperoxidase from *Caldariomyces fumago* and bromoperoxidase from *Corallina pilulifera* were compared by Itoh et al.[14].

The nucleoside antibiotic nikkomycin Z was brominated using the perhydrolase from *Streptomyces aureofaciens* Tü24[52]. Bromination occurred at the 6-position and at the 4,6-positions of the pyridine system of nikkomycin Z.

The antifungal antibiotic pyrrolnitrin [3-chloro-4-(2-nitro-3-chlorophenyl)pyrrole] was brominated at the 2-position of the pyrrole moiety by bromoperoxidase from *Streptomyces phaeochromogenes*[53]. Pyrrolnitrin was chlorinated at the 2-position and at the 2,5-positions of the pyrrole system by perhydrolases from *Pseudomonas pyrrocinia* and *Streptomyces aureofaciens*. The corresponding bromo-derivatives were also obtained with these enzymes[54].

Another phenylpyrrole compound, 2-(3,5-dibromo-2-methoxyphenyl)pyrrole was brominated to 2-bromo-, 2,3-dibromo-, 3,4-dibromo-, 2,3,4-tribromo-5-(3,5-dibromo-2-methoxyphenyl)pyrrole by perhydrolase from *Streptomyces aureofaciens* Tü24[55]. When the same substrate was chlorinated using the perhydrolases from *Pseudomonas pyrrocinia* and *Streptomyces aureofaciens* Tü24, 2-chloro-, 3-chloro-, 4-chloro-, 2,3-dichloro-, 2,4-dichloro, and 3,4-dichloro-5-(3,5-dibromo-2-methoxyphenyl)pyrrole could be isolated (Fig. 16.9-5)[56].

16.9.3.2
Halogenation of Aliphatic Compounds

Haloperoxidases catalyze the halogenation of a wide range of alkene substrates. Ethylene was iodinated, brominated, and chlorinated to the corresponding 2-haloethanol by chloroperoxidase from *Caldariomyces fumago*. Using the same enzyme and propylene as the substrate the 1-halo-2-propanols and the 2-halo-1-propanols were obtained. 1,3-Butadiene was converted into 1-bromo-3-butene-2-ol, 2-bromo-3-butene-1-ol and 1,4-dibromo-2,3-butanediol by lactoperoxidase (Fig. 16.9-6). Bromination of allene by chloroperoxidase from *Caldariomyces fumago* resulted in 2-bromo-2-propen-1-ol[57]. Propylene, allyl chloride and allyl alcohol were halogenated to yield halohydrins and dihalogenated products[58]. When several halide ions were present in the reaction mixture, heterogeneous dihalides were obtained[59]. The chlorination

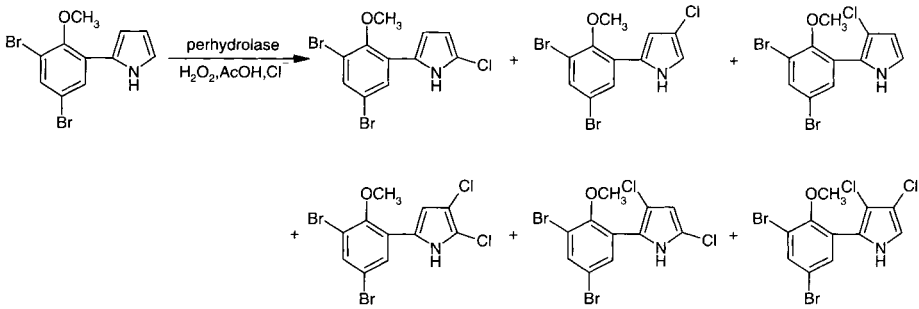


Figure 16.9-5. Chlorination of 2-(3,5-dibromo-2-methoxyphenyl)pyrrole by perhydrolase from *Streptomyces aureofaciens* Tü24[56].

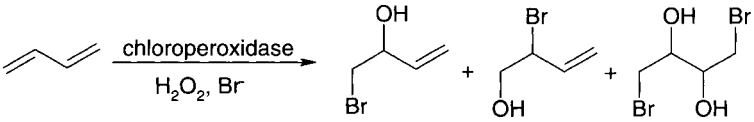


Figure 16.9-6. Bromination of 1,3-butadiene by lactoperoxidase from bovine milk[57].

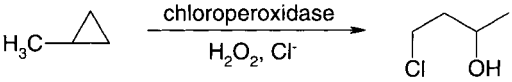


Figure 16.9-7. Chlorination of methyl cyclopropane by chloroperoxidase from *Caldariomyces fumago*[61].

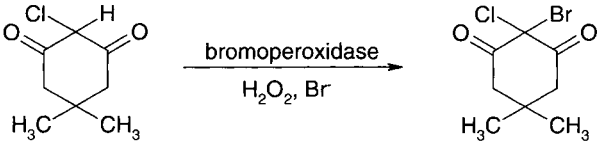


Figure 16.9-8. Bromination of monochlorodimedone, the substrate used for the search for halopereoxidases[1].

of propenylphosphonic acid resulted in the formation of 1-chloro-2-hydroxypropylphosphonic acid[60]. Phenyl acetylene was brominated to α-bromoacetophenone and α-dibromoacetophenone by lactoperoxidase. Chloroperoxidase from *Caldariomyces fumago* was used to chlorinate methyl cyclopropane to 4-chloro-2-hydroxy-butane (Fig. 16.9-7)[61].

Monochlorodimedone, the substrate used for the detection and isolation of haloperoxidases and perhydrolases (Fig. 16.9-8), and other β-diketones such as barbituric acid[62] is brominated at the 2-position by all known haloperoxidases and perhydrolases. Oxooctanoic acid and other β-ketoacids form mono- and dihalogenated ketones and carbon dioxide[63]. When β-alanine and taurine were used as substrates for myeloperoxidase the corresponding *N*-chloroamines could be detected[64–65].

As can be seen from the number of substrates halogenated by the different

haloperoxidases and perhydrolases, these enzymes show no substrate specificity. Examples of aliphatic substrates halogenated by haloperoxidases and perhydrolases are shown in Table 16.9-2.

16.9.4
Regioselectivity and Stereospecificity of Enzymatic Halogenation Reactions

16.9.4.1
$FADH_2$-dependent Halogenases

$FADH_2$-dependent tryptophan 7-halogenase shows regioselctivity which is dependent on the substrate used. With tryptophan, the enzyme is highly regioselective and catalyzes only halogenation at position 7 of the indole ring. However, with other indole derivatives halogenation occurs at positions 2 and 3 of the indole ring (Fig. 16.9-3a). Chlorination of aminophenylpyrrole derivatives by tryptophan 7-halogenase also proceeds with relaxed regioselectivity (Fig. 16-3b)[43].

Nothing is know about the regioselectivity of monodechloroaminopyrrolnitrin 3-halogenase and other $FADH_2$-dependent halogenases, but biosynthetic investigations suggest that many of these halogenases catalyze halogenation reactions regioselectively[19, 66].

So far no investigations on the stereospecificity of this type of halogenating enzymes have been reported.

Haloperoxidases show very poor regioselectivity. There are only very few reports on regioselective reactions catalyzed by haloperoxidases. Franssen et al.[62] reported the regioselective chlorination of 2-aminopyridine to 2-amino-3-chloroaminopyridine by chloroperoxidase from *Caldariomyces fumago*. When anisole was brominated using chloroperoxidase from *Caldariomyces fumago* Walter and Ballschmitter[67] found a *para*-preference for the bromination reaction with a *para : ortho* ratio of 16 compared with 9 for the normal electrophilic bromination. The *para : ortho* ratio for the chlorination of anisole with the same enzyme obtained by Brown and Hager[68] was 1.9. This discrepancy could be due to the different reaction conditions used. Walter and Ballschmitter[67] used a 50 times higher anisole concentration and only about half the amount of chloroperoxidase compared with Brown and Hager[68]. If one takes into consideration that anisole could reach the active site of the enzyme and is present at a high concentration, a relatively large part of the substrate could be chlorinated at the active site with a certain orientation and only a smaller part would be chlorinated by enzymatically produced hypochlorous acid. This effect could be amplified by smaller amounts of the enzyme and thus lower concentrations of hypohalous acid produced. However, this would mean that halogenation occurring at the active site showed a higher degree of regioselectivity, even without a specific binding site for the organic substrate.

Similar results were obtained by Itoh et al.[14, 69] for the halogenation of different substrates using bromoperoxidase from *Corallina pilulifera* and chloroperoxidase from *Caldariomyces fumago*.

In addition to poor regioselectivity, haloperoxidases also show poor stereospeci-

Table 16.9-2. Haloperoxidases used for biotransformations of aliphatic compounds and their sources.

Haloperoxidase (type)	Source	Substrate (halide)	Reference
Chloroperoxidase (heme)	*Caldariomyces fumago*	alkenes (Cl^-, Br^-, I^-)	57–60, 70, 75
		9(11)-dehydroprogesterone (Br^-)	76
		2-hydroxymethylene testosterone (Br^-)	77
		2-hydroxymethylene-17β-hydroxy-androstan-3-one (Br^-)	78
		glycals (Cl^-, Br^-, I^-)	71
		alkynes (Cl^-, Br^-, I^-)	61
		cyclopropanes (Cl^-, Br^-)	61
		β-diketones (Cl^-, Br^-)	44, 70, 79–81
Lactoperoxidase (heme)	bovine milk	alkenes (Br^-, I^-)	57, 59, 82–84
		alkynes (Br^-)	61
		cyclopropanes (Br^-)	61
Myeloperoxidase (heme)	mammals	alanine, taurine (Cl^-)	65
		β-alanine (Cl^-)	64
Bromoperoxidase (heme)	*Penicillus capitatus* *Bonnemasoinia hamifera*	α-amino acids, peptides (Br^-)	85
Bromoperoxidase (non-heme)	*Ascophyllum nodosum*	barbituric acid (Br^-)	62

ficity. Kollonitsch et al.[60] obtained optically inactive erythro-dimethyl 1-chloro-2-hydroxypropylphosphonate from *trans*-propenylphosphonic acid using chloroperoxidase from *Caldariomyces fumago*. Ramakrishnan et al.[70] investigated the bromination of racemic 2-*exo*-methylbicyclo-[2.2.1]hept-5-ene-2-*endo*-carboxylic acid to the δ-lactone and racemic bicyclo-[3.2.0]hept-2-en-6-one to the 2-*exo*-bromo-3-*endo*-hydroxybromohydrin. The products were obtained in racemic form. 2-Methyl-4-propylcyclopentane-1,3-dione was chlorinated to 2-chloro-2-methyl-4-propylcyclopentane-1,3-dione. Here the product was obtained as a 40 : 60 ratio of the racemic diastereomers. From these findings they concluded that active site chlorination by chloroperoxidase from *Caldariomyces fumago* proceeds without appreciable stereoselectivity.

On the other hand, Liu and Wong[71] described the stereoselective bromohydrations of D-galactal and L-fucal to 2-bromo-2-deoxy-D-galactose ($\beta/\alpha = 3$) and 2-bromo-2-deoxy-L-fucose ($\beta/\alpha = 2$), respectively. They also obtained the corresponding chlorinated products, however, in much lower yields.

16.9.5
Comparison of Chemical with Enzymatic Halogenation

$NADH_2$-dependent tryptophan 7-halogenase catalyzes the incoporation of a chloride atom into the indole ring at a position were direct chemical chlorination is not possible. The structures of metabolites containing halogenated indole rings suggest that similar halogenases exist which catalyze halogenation reactions at positions 2–7 of the indole ring. These enzymes are certainly very promising candidates as tools in organic synthesis, especially as they catalyze the incorporation of the halide atoms as nucleophiles, which allows regioselective and possibly stereoselective halogenation reactions.

Enzymatic halogenation catalyzed by haloperoxidases and perhydrolases involves the oxidation of halide ions to a halonium ion species which leads to the formation of hypohalous acids (Fig. 16.9-1). The products obtained by enzymatic halogenation with these enzymes are the same as the products obtained by chemical electrophilic halogenation with hypohalous acids. The differences in the *para* : *ortho* ratios in the halogenation of some aromatic compounds could be due to a mixture of halogenation at or near the active site and in solution.

The major advantage of enzymatic halogenation using haloperoxidases and perhydrolases is that the enzymes have a very low substrate specificity and that no free halogen is needed which makes halogenation catalyzed by these enzymes less hazardous than chemical halogenation.

Some of the non-heme haloperoxidases and perhydrolases are very stable, even against organic solvents, and easy to use as they do not need any cofactors. However, care has to be taken not to use too high concentrations of hydrogen peroxide, as this could lead to oxidation of the substrate.

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17
Isomerizations

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17.1
Introduction

Isomerases catalyze the isomerization of substrates, and are classified into five groups as follows:

Racemases and epimerases (E. C. class 5.1)
They are defined as enzymes that catalyze the isomerization of a substrate through stereochemical reverse rearrangement of a substituent bound to a chiral center (usually a chiral carbon) in the substrate molecule. Racemases act on molecules containing only the asymmetric center concerned in the reaction. Epimerases act on substrates containing one or more asymmetric centers in addition to the reactive chiral center.

cis-trans-Isomerases (E. C. class 5.2)
They catalyze the interconversion of *cis-trans* geometrical isomers.

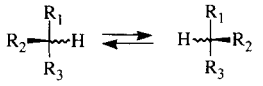
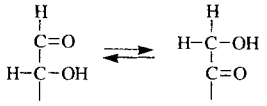
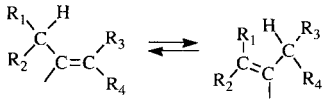
Sugar isomerases, tautomerases, Δ-isomerases, etc. (E. C. class 5.3)
Sugar isomerases catalyze the interconversion between aldose and ketose. Tautomerases catalyze a keto-enol tautomerization. Δ-Isomerases catalyze the shift of a double bond. The reactions catalyzed by these enzymes proceed through intramolecular oxidation and reduction.

Mutases (E. C. class 5.4)
They catalyze the transfer of a substituent to produce a structural isomer.

Cycloisomerases (E. C. class 5.5)
They catalyze the ring formation through an intramolecular lyase reaction.

Isomerizations catalyzed by most of these enzymes proceed through 1,1-, 1,2-, or 1,3-hydrogen shifts (Table 17-1), while mutases catalyze exchange of a hydrogen

Table 17-1. Enzyme-catalyzed isomerizations classified as hydrogen shifts.

Type	Examples	Category
1,1-Shifts		Epimerases, Racemases
1,2-Shifts		Aldose-ketose isomerases
1,3-Shifts		Allylic isomerizations

atom with particular functional groups such as amino, hydroxy, and α-amino-α-carboxymethyl groups attached at neighboring carbon atoms of the substrates through homolytic cleavage.

Here we describe enzymological properties of representative racemases, epimerases, and isomerases, and their application to production of various optically active compounds.

17.2 Racemizations and Epimerizations

Since the discovery of enzymatic racemization of lactate by lactic acid bacteria[1, 2], *Clostridium acetobutyricum*[3], and *Cl. butyricum*[4], a variety of racemases and epimerases have been demonstrated, and they are classified into the four groups as follows:

- Amino acid racemases and epimerases catalyzing racemization and epimerization at the chiral center containing an NH_2 or NH group (E.C. class 5.1.1);
- Mandelate racemase, lactate racemase, and others acting at the chiral center containing an OH group (E.C. class 5.1.2);
- Various carbohydrate epimerases such as UDP-D-glucose-4'-epimerase (E.C. class 5.1.3);
- Methylmalonyl CoA epimerase and some others, in whose substrates a CH_3 group is bound to the chiral centers (E.C. class 5.1.99).

Racemases and epimerases have been used for production of various optically active compounds from cheaply-available racemic substrates by combination of enzymes that act specifically on one of the isomers of the racemates to catalyze hydrolysis, oxidation, reduction, elimination, replacement, and other reactions. The racemases and epimerases used act exclusively on the substrates, but not on the products of the

reaction. Thus, total conversion of the racemic substrates into the desired optically-active compounds is achieved. Here we describe enzymological characteristics of the representative racemases and epimerases, and their application to production of optically active compounds.

17.2.1
Pyridoxal 5'-phosphate-dependent Amino Acid Racemases and Epimerases

17.2.1.1
Alanine Racemase (E.C. 5.1.1.1)

Alanine racemase is a bacterial enzyme that catalyzes racemization of L- and D-alanine, and requires pyridoxal 5'-phosphate (PLP) as a cofactor. The enzyme plays an important role in the bacterial growth by providing D-alanine, a central molecule in the peptidoglycan assembly and cross-linking, and has been purified from various sources[5–16]. The enzyme has been used for the production of stereospecifically deuterated NADH and various D-amino acids by combination of L-alanine dehydrogenase (E.C. 1.4.1.1), D-amino acid aminotransferase (E.C. 2.6.1.21), and formate dehydrogenase (E.C. 1.2.1.2)[17, 18].

17.2.1.1.1 Gene Cloning and Primary Structure

Two distinct alanine racemase genes were cloned from the *Salmonella typhimurium* chromosome. One mapped at minute 37 on the chromosome is termed *dad*B, and the other mapped at minute 91 is termed the *alr* gene[19]. The *dad*B alanine racemase is formed inducibly and functions in the catabolism of L-alanine: the *alr* enzyme is synthesized constitutively, and functions in the anabolic assembly of peptidoglycan[19]. Alanine racemase genes were also cloned from *Bacillus stearothermophilus*[10], *Bacillus subtilis*[20], *Bacillus psychrosaccharolyticus*[14], and *Aquifex pyrophilus*[15]. Two distinct alanine racemase genes were assigned in the genome sequences of *Escherichia coli*[12], *B. subtilis*, *Pseudomonas aeruginosa*, and *Vibrio cholerae*, but only a single one occurs in the other bacterial genomes whose complete nucleotide sequences were determined as shown at internet sites such as http://www.genome.ad.jp/kegg/catalog/org_list.html.

Uo et al.[16] have found that fission yeast, *Schizosaccharomyces pombe*, has also the alanine racemase gene, which is involved in the catabolism of D-alanine in *S. pombe* in the same manner as *dad*B of *S. typhimurium*. The yeast enzyme only shows any high degree of similarity to the alanine racemases of γ-proteobacteria (gram-negative phylum). Therefore, the gene of *S. pombe* has possibly been acquired from γ-proteobacteria through some events of horizontal gene transfer such as conjugation: *S. pombe* is known to be a recipient of the genes from *E. coli* through direct conjugation.

D-Alanine occurs in various natural compounds produced by fungus. For example, cyclosporin A contains D-alanine as a component and is produced by *Tolypocladium niveum*[12]. Alanine racemase is involved in the biosynthesis of D-alanine in this fungus.

17.2.1.1.2 Stability

The native *dad*B and *alr* racemases from *Salmonella typhimurium* are readily inactivated by digestion with α-chymotrypsin, trypsin, and subtilisin[22]. However, the *Bacillus stearothermophilus* enzyme is stable even after fragmentation into two pieces[23, 24]. *A. pyrophilus*, a hyperthermophilic bacterium, produces extremely stable alanine racemase[15]. It maintains catalytic activity in the presence of organic solvents as well. On the other hand, *Bacillus psychrosaccharolyticus*, a psychrophyilic bacterium, produces a thermo-labile enzyme[14]. However, it shows high catalytic activity at low temperatures, such as at 0 °C. Similar cold activity and thermal instability was found in the enzyme from a psychrophile isolated from raw milk, *Pseudomonas fluorescens*[11].

17.2.1.1.3 Reaction Mechanism

Reaction of alanine racemase proceeds through the steps shown in Fig. 17-1. PLP bound with the active-site lysyl residue (A) reacts with a substrate to form an external Schiff base (B) through transaldimination. The subsequent α-hydrogen abstraction

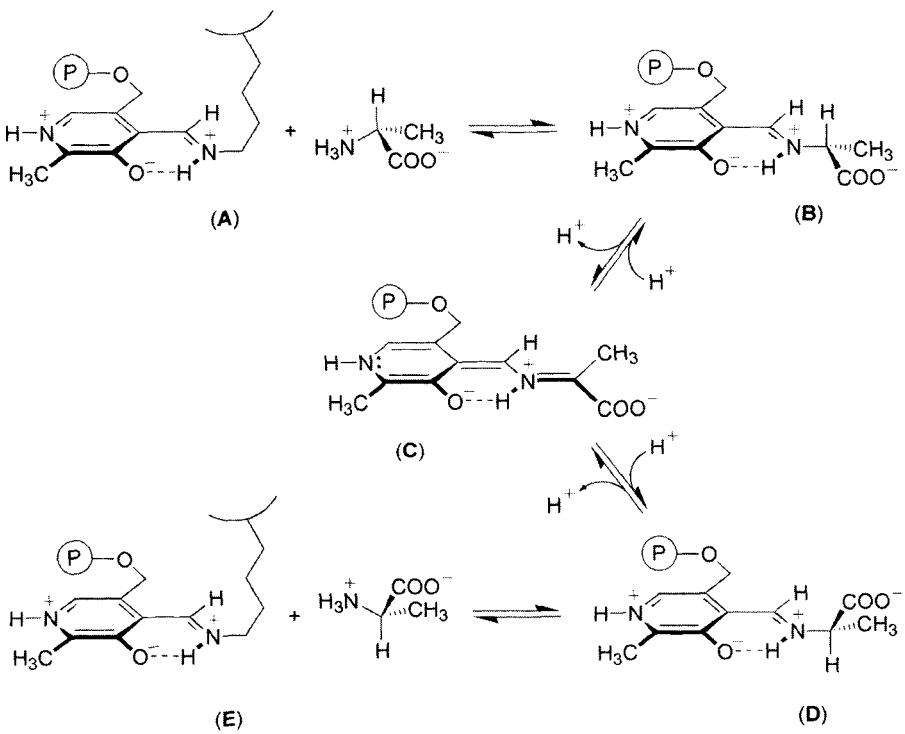


Figure 17-1. Mechanism of the alanine racemase reaction. A, An internal aldimine of PLP with a lysyl residue; B, an external aldimine of PLP with D-alanine; C, a quinonoid intermediate formed after removal of a hydrogen from alanyl external aldimines B or D; D, an external aldimine of PLP with L-alanine. Reprinted from Watanabe et al.[33].

results in the formation of a resonance-stable deprotonated intermediate (C). If reprotonation occurs at the α-carbon of the substrate moiety on the opposite face of the planar intermediate (C), then an antipodal aldimine (D) is formed. The ε-amino group of the lysine residue is substituted for the isomerized amino acid through transaldimination, and the internal aldimine (A) is regenerated. According to Dunathan[25], the C^{α}-H - bond to be broken is positioned perpendicularly to the plane of the conjugated π-system of the external Schiff base intermediate, in order to achieve maximum orbital overlap with the π electron system of the complex, resulting in a substantial rate enhancement for the cleavage of that bond.

The racemization reaction proceeds via either a one-base[26] or two-base[27] mechanism. The one base mechanism is characterized by the retention of the substrate-derived proton in the product (internal return)[26]. By this criterion, reactions catalyzed by α-amino-ε-caprolactam racemase[28] and amino acid racemase with low substrate specificity (E.C. 5.1.1.10)[26] have been considered to proceed through the one-base mechanism. However, such internal returns were not observed in the reactions catalyzed by alanine racemases from *E. coli*[26], *Bacillus stearothermophilus*[29], and *Salmonella typhimurium* (*dad*B and *alr*)[29]. The internal return is not expected to occur in the reactions of two-base mechanism. In fact, kinetic analyses[30] indicated that the alanine racemase reaction proceeds through a two-base mechanism: proton donors and proton acceptors are situated on both sides of the planar intermediate (Fig. 17-1, C) and accomplish removal and return of the α-hydrogen of the substrate amino acid. X-ray crystallographic studies[31, 32] suggested that Lys 39 and Tyr 265 of alanine racemase from *B. stearothermophilus* serve as the bases (Fig. 17-2). Watanabe *et al.*[33] showed that the lysyl residue binding PLP in the racemase (Lys 39) acts as the base catalyst specific to the D-enantiomer of alanine. The crystal structure of the enzyme complex with *R*-1-aminoethylphosphonic acid[32], a tight-bind inhibitor of the enzyme[34], demonstrated that the phenolic oxygen of Tyr 265 is appropriately aligned for proton abstraction from an L-isomer in the active site of the structure: Tyr 265 is the second base specifically acting on the L-alanyl-PLP aldimine.

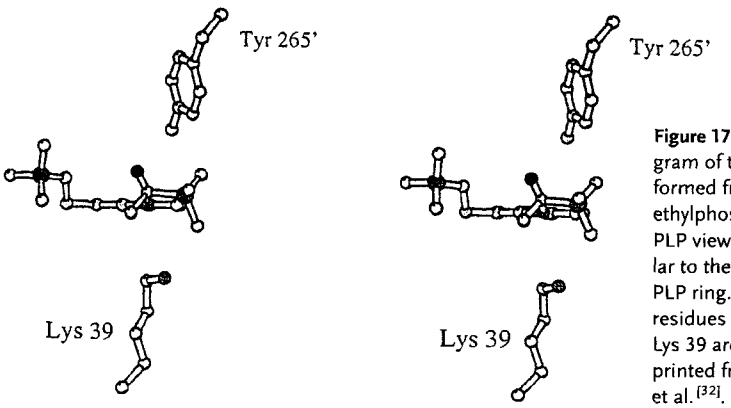


Figure 17-2. Stereodiagram of the aldimine formed from 1-aminoethylphosphonate and PLP viewed perpendicular to the plane of the PLP ring. The catalytic residues Tyr 265' and Lys 39 are shown. Reprinted from Stamper et al.[32].

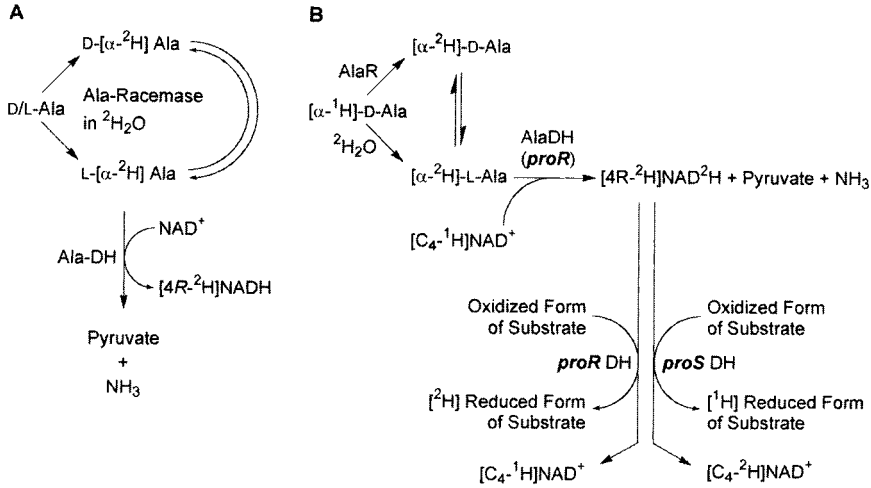


Figure 17-3. A, Preparation of [4*S*-^{2}H]-NADH by coupling of alanine racemase and L-alanine dehydrogenase. B, *In situ* determination of stereospecificity of H-transfer by ^{1}H-NMR. AlaR, AlaDH, and DH represent alanine racemase, L-alanine dehydrogenase, and dehydrogenase, respectively.

17.2.1.1.4 Production of Stereospecifically Deuterated NADH

NAD-linked dehydrogenases show either pro-*S* or pro-*R*-stereospecificity for hydrogen removal from the C4 position of the nicotinamide moiety of the reduced coenzymes. The stereospecificity of hydrogen transfer is examined by means of stereospecifically C4-deuterated NADH, which is prepared enzymatically from NAD^+ and deuterated substrates by tedious procedures.

Esaki et al. developed a simple method to produce the stereospecifically deuterated NADH by an NAD^+-dependent dehydrogenase by combination with amino acid racemase[35]. L-Alanine dehydrogenase transfers deuterium of [2-^{2}H]-L-alanine to NAD^+ to produce [4*R*-^{2}H]-NADH[36]. Alanine racemase catalyzes the C2-deuteration of D and L-alanine in ^2H_2O[10], and [4*R*-^{2}H]-NADH was produced from D-alanine and NAD^+ by coupling of the reactions catalyzed by alanine racemase and L-alanine dehydrogenase in ^2H_2O (Fig. 17-3A). Furthermore, this finding led to development of a simple procedure for the *in situ* analysis of stereospecificity of hydrogen transfer of NADH by an NAD-dependent dehydrogenase by means of ^{1}H-NMR (Fig. 17-3B)[35].

17.2.1.1.5 Production of D-Amino Acids

Considerable attention has been paid to multi-enzyme reaction systems as a means to the stereospecific production of L-amino acids[37]. Wichmann et al. have developed a continuously operated membrane reactor for production of L-leucine from α-ketoisocaproate[38]. The system is also applicable to the production of several other aliphatic L-amino acids such as L-valine, L-*tert*-leucine and [^{15}N]-L-leucine. The

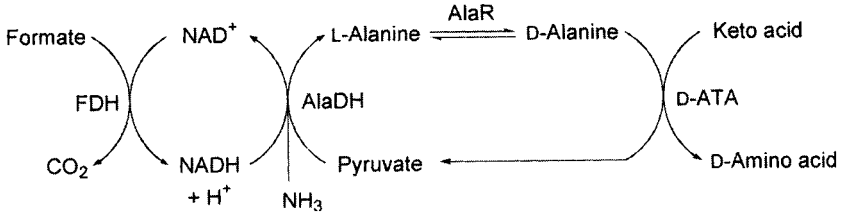


Figure 17-4. Synthesis of D-amino acids from α-keto acid, formate, NAD^+, D-alanine, and ammonia by coupling of L-alanine dehydrogenase (AlaDH), formate dehydrogenase (FDH), alanine racemase (AlaR), and D-amino acid aminotransferase (D-ATA).

process has been successfully scaled-up for industrial production of these L-amino acids[39]. A similar system has been developed for production of L-phenylalanine[40, 41] and L-β-chloroalanine[40–43]. However, little attention has been paid to the stereospecific production of D-amino acids by means of multi-enzyme reaction systems, although D-amino acids have been paid considerable attention[44]. For example, substantial amounts of D-serine, D-aspartate and other D-amino acids occur in mammalian brain[45–47], and ^{13}N-labeled D-amino acids are expected to be useful for the study of their metabolism in brain[48].

A simple procedure was established for the synthesis of various D-amino acids by means of four types of thermostable enzymes: alanine racemase, D-amino acid aminotransferase[49, 50], L-alanine dehydrogenase[5], and formate dehydrogenase (Fig. 17-4)[17]. The commercial preparation of formate dehydrogenase from *Candida boidinii* used by Wichmann et al.[38] is not sufficiently stable. However, Galkin et al.[52] cloned and expressed the gene of thermostable formate dehydrogenase in *E. coli*.

D-Phenylalanine and D-tyrosine, which are the poor substrates for D-amino acid aminotransferase, were synthesized in an optical purity of essentially 100%, but with yields of lower than 50%. However, the yields were increased by addition of excess amounts of the D-amino acid aminotransferase (Table 17-2)[17]. Selenium is an essential micronutrient for mammals, fish and several bacteria, although it is toxic at a high concentration[52, 53]. D-Selenomethionine was produced in an 80% yield based on 2-oxo-4-methylselenobutyrate[54]. Norvaline, valine, and α-aminobutyrate were also produced with high yields. However, α-aminobutyrate was synthesized as a racemic mixture. D-Norvaline was obtained at an enantiomeric excess of only 30%. The low optical purity is probably due to the action of L-alanine dehydrogenase: α-ketobutyrate and α-ketovalerate are reduced by L-alanine dehydrogenase at rates of 79 and 6.6% relative to that of pyruvate, respectively[10]. Moreover, alanine racemase also racemizes α-aminobutyrate and norvaline, though very slowly[55]. Thus, this method is not applicable to the stereospecific production of D-α-aminobutyrate and D-norvaline. The preparations of D-valine, D-methionine and D-norleucine also suffered contamination by the antipodes at concentrations of 4, 3, and 1%, respectively, due to the action of L-alanine dehydrogenase on the α-keto analogs of these amino acids[51]. However, D-glutamate, D-phenylalanine and D-tyrosine were efficiently produced in the system. The final concentration of D-glutamate produced

Table 17-2. Synthesis of D-amino acids from α-keto acids by combination of four purified enzymes: alanine racemase, L-alanine dehydrogenase, formate dehydrogenase, and D-amino acid aminotransferase.

Substrate	Product	Yield (%)[a]	ee (%)
α-Ketoglutarate	D-glutamate	98	100
α-Ketoisocaproate	D-leucine	80	>99
α-Ketocaproate	D-norleucine	82	98
α-Keto-γ-thiomethylbutyrate	D-methionine	95	94
α-Ketoisovalerate	D-valine	90	92
α-Ketovalerate	D-norvaline	92	30
α-Ketobutyrate	α-aminobutyrate	93	0
Phenylpyruvate	D-phenylalanine	72[b]	100
Hydroxyphenylpyruvate	D-tyrosine	70[b]	100

a The yields were determined after an 8 h incubation.
b The amount of D-amino acid aminotransferase used (30 units) was 10-fold higher than that in other systems

was only around 0.3 M, limited because of the equilibrium of the D-amino acid aminotransferase reaction. The method is most suitable for stereospecific conversion of α-keto acids into the corresponding D-amino acids, in particular labeled compounds, for example with ^{13}N by means of $^{13}N\text{-}NH_3$.

The industrial use of the above-mentioned systems depends predominantly on the cost of the enzymes, although the intact cells of microorganisms containing the enzymes can be used as catalysts in order to decrease costs[56]. In most cases, however, additional genetic improvements through metabolic engineering are required, thereby new functional combinations are made by the rational transfer of pathways from one organism to another[57]. The transfer of the ethanol pathway from *Zymomonas mobilis* to other enteric bacteria represents an example of this approach[58]. In the above-mentioned system, various D-amino acids can be produced from the corresponding α-keto acids, if four functional genes are introduced into one microorganism. The simultaneous expression of all enzymes in a single cell

Table 17-3. Synthesis of D-amino acids from α-keto acids by *E. coli* cells harboring pFADA which codes for four enzyme genes: alanine racemase, L-alanine dehydrogenase, formate dehydrogenase, and D-amino acid aminotransferase.

Substrate	Product	Yield (%)[a]	ee (%)
α-Ketoglutarate	D-glutamate	85	100
α-Ketoisocaproate	D-leucine	76	>99[b]
α-Ketocaproate	D-norleucine	70	88
α-Keto-γ-thiomethylbutyrate	D-methionine	80	90
α-Ketoisovalerate	D-valine	85	92
α-Ketovalerate	D-norvaline	90	35
α-Ketobutyrate	α-aminobutyrate	95	0
Phenylpyruvate	D-phenylalanine	15	ND[c]
Hydroxyphenylpyruvate	D-tyrosine	5	ND

a The yields were determined after a 12 h incubation
b The optical purity determined by HPLC is >99.9%
c ND, not determined.

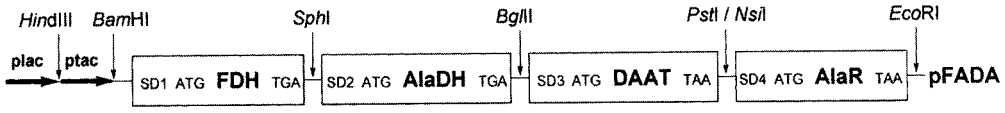


Figure 17-5. Construction of the plasmid used for the production of D-amino acids by expression in *E. coli* cells; formate dehydrogenase (FDH), L-alanine dehydrogenase (AlaDH), alanine racemase (AlaR), and D-amino acid aminotransferase (DAAT).

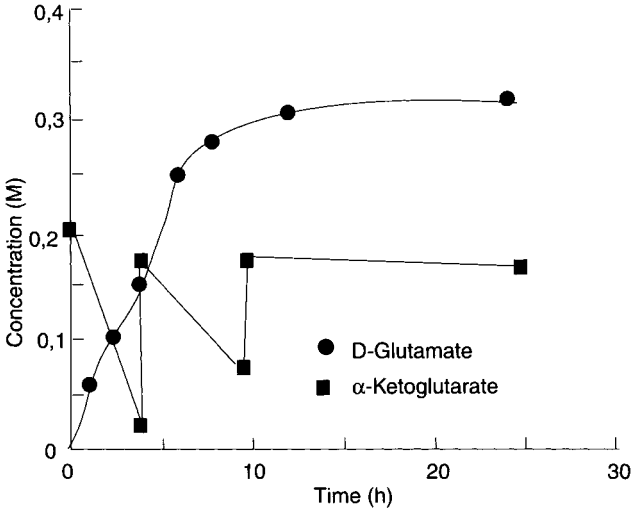


Figure 17-6. Time course for the production of D-glutamate with *E. coli* cells containing pFADA. α-Ketoglutarate was added after 4 and 10 h of incubation at a final concentration of approximately 0.2 M.

provides additional benefit for industrial applications: the intracellular pool of NAD^+ (supplied by the cell itself) could be used for NADH regeneration without any additional supplies.

Galkin et al.[18] constructed plasmids containing, in addition to the thermostable formate dehydrogenase gene, all three genes required for the synthesis of D-amino acids (Fig. 17-5). D-Enantiomers of glutamate and leucine were produced at high optical purity and high conversion rates with the recombinant *E. coli* cells harboring the plasmid for coding of the four heterologous genes (Table 17-3). α-Keto acids, particularly branched-chain and long-chain α-keto acids, are toxic, inhibiting the growth of *E. coli* when added at concentrations of only 15–30 mM. Therefore, Galkin et al. used the resting cells of the recombinant *E. coli* instead of growing ones. Moreover, the isolation of products in the resting-cell system is much easier than when using growth media containing complex ingredients such as yeast extracts. The final concentration of D-glutamate produced was around 0.3 M (Fig. 17-6).

17.2.1.2
Amino Acid Racemase with Low Substrate Specificity (E.C. 5.1.1.10)

An amino acid racemase which shows very broad substrate specificity was discovered in *Pseudomonas striata* (= *Ps. putida*), purified, and characterized[59]. The enzyme catalyzes racemization of various amino acids except aromatic and acidic

amino acids. A similar enzyme also occurs in *Aeromonas punctata*[60]. Arginine racemase, which also shows a broad substrate specificity, has been demonstrated in *Pseudomonas graveolens* (= *Pseudomonas taetrolens*)[61]. These amino acid racemases do not act on threonine, valine and their analogs, whose β-methylene group is substituted. Recently, Lim et al.[62] found, in *Ps. putida* ATCC 17642, a new amino acid racemase catalyzing not only racemization of various amino acids but also epimerization of D- and L-threonine by stereoconversion at the α-position: it catalyzes epimerization of L-to D-allo- and also of D- to L-*allo*-threonine.

Amino acid racemase with low substrate specificity catalyzes racemization of leucine and various other amino acids, which are also α-deuterated in ^2H_2O during their racemization[63]. Therefore, [4*S*-^{2}H]-NADH was produced in the same manner as described above with the racemase and L-leucine dehydrogenase (E.C. 1.4.1.9), which is pro-*S* specific[35].

Amino acid racemase with low substrate specificity of *Ps. putida* ATCC 17642 does not racemize aromatic and acidic amino acids. However, phenylalanine and phenylglycine undergo α-hydrogen exchange with deuterium from the solvent when incubated with the racemase in ^2H_2O. Lim et al.[64] found that each enantiomer of both α-deuterated phenylalanine and phenylglycine are produced stereospecifically with retention of the C2 configuration. This α-hydrogen exchange reaction is applicable to the production of α-deuterated phenylalanine and phenylglycine.

Makiguchi and coworkers established a method to synthesize L-tryptophan from D,L-serine and indole by means of tryptophan synthase (E.C. 4.2.1.20) from *E. coli* and the amino acid racemase with low substrate specificity of *Ps. striata* (= *Ps. putida*)[65]. Both D,L-serine and indole are cheaply available by chemical synthesis. Tryptophan synthase catalyzes the β-replacement reaction of L-serine with indole to produce L-tryptophan, and the amino acid racemase with low substrate specificity converts unreacted D-serine into L-serine. Because the racemase does not act on tryptophan, almost all D,L-serine is converted into optically pure L-tryptophan. Makiguchi et al.[65] succeeded in producing L-tryptophan in a 200 L reactor using intact cells of *E. coli* and *Ps. putida*[65]. Under the optimal conditions established, 110 g L^{-1} of L-tryptophan was formed in molar yields of 91 and 100% for added D,L-serine and indole, respectively, after 24 h of incubation with intermittent indole feeding. Continuous production of L-tryptophan was also achieved using immobilized cells of *E. coli* and *Ps. putida*. The maximum concentration of L-tryptophan formed was 5.2 g L^{-1} (99% molar yield for indole).

S-Adenosyl-L-methionine is the important methyl donor in biological transmethylation to form *S*-adenosyl-L-homocysteine, which is hydrolyzed to adenosine and homocysteine by *S*-adenosyl-L-homocysteine hydrolase (E.C. 3.3.1.1) *in vivo*. However, equilibrium of the *S*-adenosyl-L-homocysteine hydrolase reaction favors the direction toward synthesis of *S*-adenosyl-L-homocysteine. Shimizu et al. developed a simple and efficient method for the high yield preparation of *S*-adenosyl-L-homocysteine with *S*-adenosyl-L-homocysteine hydrolase of *Alcaligenes faecalis*, in which the cellular content of *S*-adenosyl-L-homocysteine hydrolase was about 2.5% of the total soluble protein. *S*-Adenosyl-L-homocysteine was produced at a concentration of about 80 g L^{-1} with a yield of nearly 100%[66]. However, when racemic

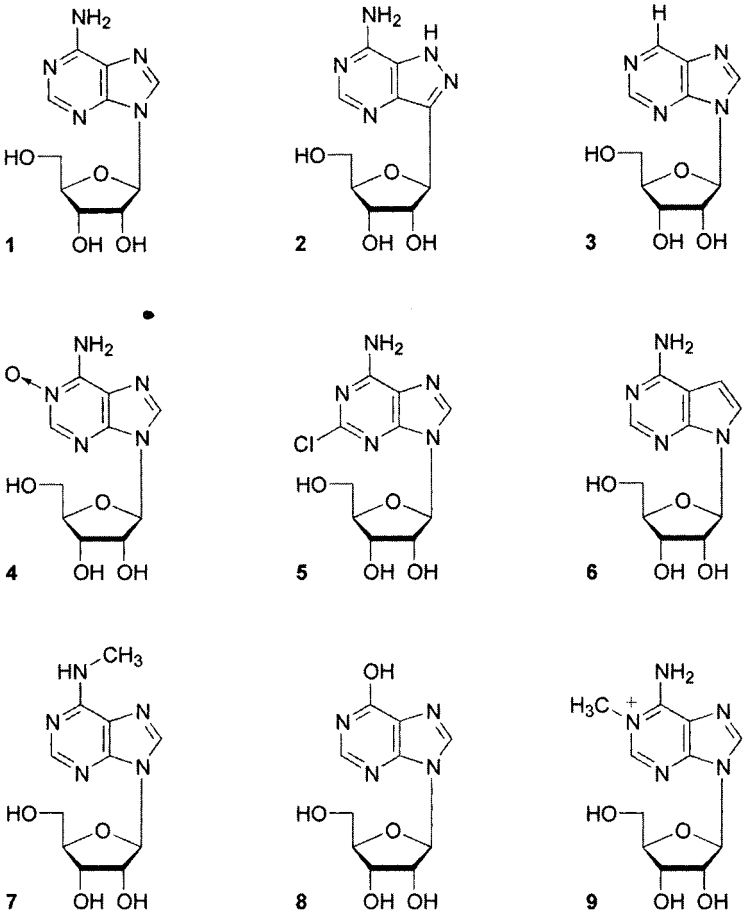


Figure 17-7. Structures of adenosine and related nucleosides which serve as substrates for *S*-adenosyl-L-homocysteine hydrolase. 1, Adenosine; 2, formycin A; 3, neburalin; 4, adenosine N^1-oxide; 5, 2-chloroadenosine; 6, tubercidine; 7, N^6-methyladenosine; 8, inosine; 9, 1-methyladenosine.

homocysteine was used, the D-enantiomer remained unreacted. When *Ps. striata* (= *Ps. putida*) cells were used as the catalyst, D-homocysteine was converted into *S*-adenosyl-L-homocysteine: the amino acid racemase with low substrate specificity acts on homocysteine, but not on *S*-adenosylhomocysteine. *A. faecalis* is better than *Ps. striata* in showing higher *S*-adenosyl-L-homocysteine hydrolase and lower adenosine deaminase activities than those of *Ps. striata*. Therefore, a mixture of both bacterial cells was used to produce 70 g L^{-1} of *S*-adenosyl-L-homocysteine from D,L-homocysteine and adenosine with a molar yield of nearly 100%[66]. *S*-Adenosyl-L-homocysteine hydrolase acts on various adenosine analogs, and the corresponding *S*-nucleotidyl-L-homocysteines (Fig. 17-7) were synthesized from the analogs and D,L-homocysteine by means of both bacterial cells[67].

17.2.1.3
α-Amino-ε-caprolactam Racemase

α-Amino-ε-caprolactam (ACL) is a chiral heterocyclic compound synthesized from cyclohexene, which is a by-product in the industrial production of nylon. Fukumura[68–70] established an enzymatic method to produce L-lysine from D,L-ACL. The process is composed of two enzyme reactions: the selective hydrolysis of L-ACL to L-lysine, and the racemization of ACL (Fig. 17-8). The L-ACL-hydrolyzing enzyme (α-amino-ε-caprolactam hydrolase (E.C. class 3.5.2) is distributed in the cells of *Cryptococcus laurentii* and other yeasts[68–70], and its synthesis is induced by D,L-ACL. The enzyme purified to homogeneity from a cell extract of *C. laurentii* has a molecular weight of about 185 000, and is activated by $MnCl_2$ and $MgCl_2$[71]. L-ACL is the only substrate of the hydrolase: D-ACL and ε-caprolactam are not hydrolyzed.

ACL racemase has been found in the cells of *Achromobacter obae* and other bacteria[72], and is a unique enzyme among racemases in acting exclusively on cyclic amides derived from α,ω-diamino acids. Ahmed et al.[73] purified the enzyme to homogeneity from the cell extract of *A. obae*, and characterized it. The enzyme is composed of a single polypeptide chain whose molecular weight is about 50 000, and contains 1 mol of PLP per mol of enzyme as a coenzyme. In addition to both isomers of ACL, D- and L-α-amino-δ-valerolactam also serve as effective substrates[74]. The enzyme catalyzes the exchange of the α-hydrogen of the substrate with deuterium or tritium during racemization in deuterium oxide or tritium oxide[28]. By tritium-incorporation experiments, the enzyme was shown to catalyze both inversion and retention of configuration of the substrate with a similar probability in each turnover. When [α-^{2}H]-D-ACL and unlabeled D-ACL were converted into the L-isomer by ACL racemase in water and in deuterium oxide, respectively, in the presence of excess L-ACL hydrolase, α-hydrogen (or α-deuterium) was retained significantly in the product[28]. Therefore, a single base mechanism has been proposed for the racemization catalyzed by ACL racemase. The ACL racemase gene has been cloned from the chromosomal DNA of *A. obae*, and its complete nucleotide sequence determined, which revealed that the enzyme consists of 435 amino acids and that its molecular weight is 45 568[75].

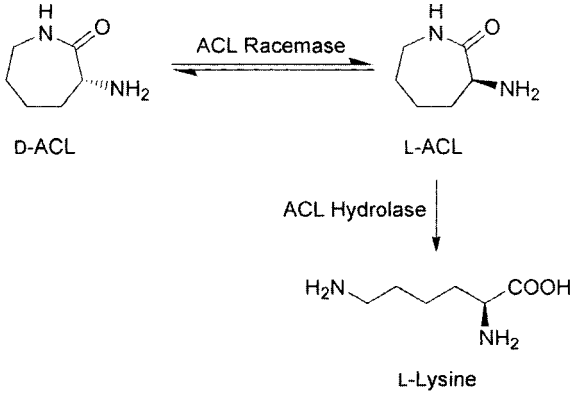


Figure 17-8. Total conversion of racemic ACL into L-lysine by coupling of ACL racemase and ACL hydrolase reactions.

17.2.2
Cofactor-independent Racemases and Epimerases Acting on Amino Acids

17.2.2.1
Glutamate Racemase (E.C. 5.1.1.3)

D-Glutamate as well as D-alanine is an important component of the peptidoglycan of bacterial cell walls[76], and is produced by glutamate racemase[77, 78]. Lactic acid bacteria show high activity of the enzyme[79], and glutamate racemase was first purified from *Pediococcus pentosaceus*[80, 81].

17.2.2.1.1 Gene Cloning

Nakajima et al. cloned the glutamate racemase gene of *Pediococcus pentosaceus*[80]. Glutamate racemase genes have been also cloned from various other sources: *Lactobacillus fermenti*[82, 83], *Lactobacillus brevis*[84], *E. coli*[85], *Bacillus pumilus*[86], *Aquifex pyrophilus*[87], and *Bacillus subtilis*[88, 89].

17.2.2.1.2 Enzymological Properties

The glutamate racemase gene from *Pediococcus pentosaceus* was over-expressed in the recombinant cells, but formed an inclusion body[81]. However, the enzyme was solubilized with 6 M urea, renatured by dialysis to remove urea, and purified to homogeneity with a high overall yield[81]. The amount of enzyme produced by the clone cells corresponded to about 38% of the total insoluble proteins. However, the glutamate racemase gene was solubilized *in vivo* in an active form when it was co-expressed with the gene of chaperonin GroESL[90]. Choi et al. isolated the active enzyme and purified it effectively[81]. The enzyme is composed of a subunit with a molecular mass of about 29 kDa. The enzyme acts specifically on glutamate with K_M values of 14 and 10 mM for D- and L-glutamates, respectively. None of other amino acids occurring in proteins including aspartate, asparagine, and glutamine are racemized. Other glutamate analogs (homocysteate, α-aminoadipate, glutamate γ-methyl ester, *N*-acetylglutamate, α-hydroxyglutarate, and cysteine sulfinate) are also inert. However, L-homocysteine sulfinate, a γ-sulfinate analogue of glutamate, is racemized at a rate of about 10% of that of L-glutamate. Amino acid racemases generally require PLP as a cofactor, but glutamate racemase is dependent on neither PLP nor on any other cofactor[80, 91]. Proline racemase (E.C. 5.1.1.4)[92], diaminopimelate epimerase (E.C. 5.1.1.7)[93] and hydroxyproline epimerase (E.C. 5.1.1.8)[94] also require no coenzyme.

Glutamate racemase from *E. coli* is unique because it is activated about 100 fold in the presence of UDP-*N*-acetylmuramoyl-L-alanine (UDP-MurNAc-L-Ala), the precursor of peptidoglycan[95]. UDP-MurNAc-L-Ala is ligated to D-glutamate, a product of the glutamate racemase reaction, by the catalysis of UDP-*N*-acetylmuramoyl-L-alanyl-D-glutamate synthetase (E.C. 6.3.2.9). Thus, the activation of the *E. coli* glutamate racemase by UDP-MurNAc-L-Ala has a physiological importance in the

regulation of peptidoglycan biosynthesis[95]. In contrast, glutamate racemases of Gram-positive bacteria such as *Lactobacillus fermenti*, *Lactobacillus brevis*, *Bacillus pumilus* are not activated by UDP-MurNAc-L-Ala, though these enzymes show about 30% sequence similarities to the *E. coli* enzyme. The predominant difference between the *E. coli* enzyme and the glutamate racemases of the Gram-positive bacteria is that the former has a 21-amino acid extension at the *N*-terminus as compared with the latter enzymes: the *N*-terminal region is responsible for the activation[95].

Glutamate racemase produced in cell extracts of *Bacillus subtilis*, an abundant producer of poly-γ-glutamate, is a monomer with a molecular mass of about 30 kDa containing no cofactor[88]. It almost exclusively catalyzes racemization of glutamate and is mainly concerned in D-glutamate synthesis for poly-γ-glutamate production. *B. subtilis* produces another isozyme of glutamate racemase encoded by the *YrpC* gene[89]. Ashiuchi et al. cloned both enzyme genes and compared their enzymological properties[88, 89]. Enzymological properties of YrpC, such as the substrate specificity and optimum pH, are similar to those of the other glutamate racemase (Glr). The thermostability of YrpC, however, is considerably lower than that of Glr. In addition, YrpC shows higher affinity and lower catalytic efficiency for L-glutamate than Glr[89].

17.2.2.1.3 Structure and Mechanism

Glutamate racemase contains one essential cysteine residue per mol of enzyme, whose chemical modification results in complete inactivation[91]. Choi et al. determined the amount of tritium incorporated into the substrate and product enantiomer during incubation with the enzyme in tritium water, and found that tritium is exclusively incorporated into the product enantiomer regardless of the configuration of the substrate used[91]. This is compatible with a model in which two different bases participate in abstraction and return of α-hydrogen of the substrate. One of the two bases involved in catalysis is suggested to be the essential cysteine residue: a thiolate from one of the cysteines abstracts the α-proton, and the other cysteine thiol delivers a proton to the opposite face of the resulting carbanionic intermediate[91].

Kim et al.[87] cloned the glutamate racemase gene from *Aquifex pyrophilus*, a hyperthermophilic bacterium, and expressed it in *E. coli*. The enzyme shows strong thermostability in the presence of phosphate ion, and it retains more than half of its original activity after incubation at 85 °C for 90 min. Hwang et al.[96] crystallized the glutamate racemase of *A. pyrophilus* and determined the tertiary structure of the enzyme by X-ray crystallography. The enzyme is composed of two identical subunits, and each monomer consists of two α/β fold domains. Hwang et al. has also proposed a mechanism in which two cysteine residues are involved in the catalysis (Fig. 17-9)[96].

Glavas and Tanner replaced the two cysteine residues, Cys 73 and Cys 184, by serine, and analyzed the reactions catalyzed by the mutant enzymes: the elimination of water from a substrate analog, *N*-hydroxyglutamate, through a one-base requiring reaction[97]. The C73S mutant was a much poorer catalyst than the wild-type enzyme

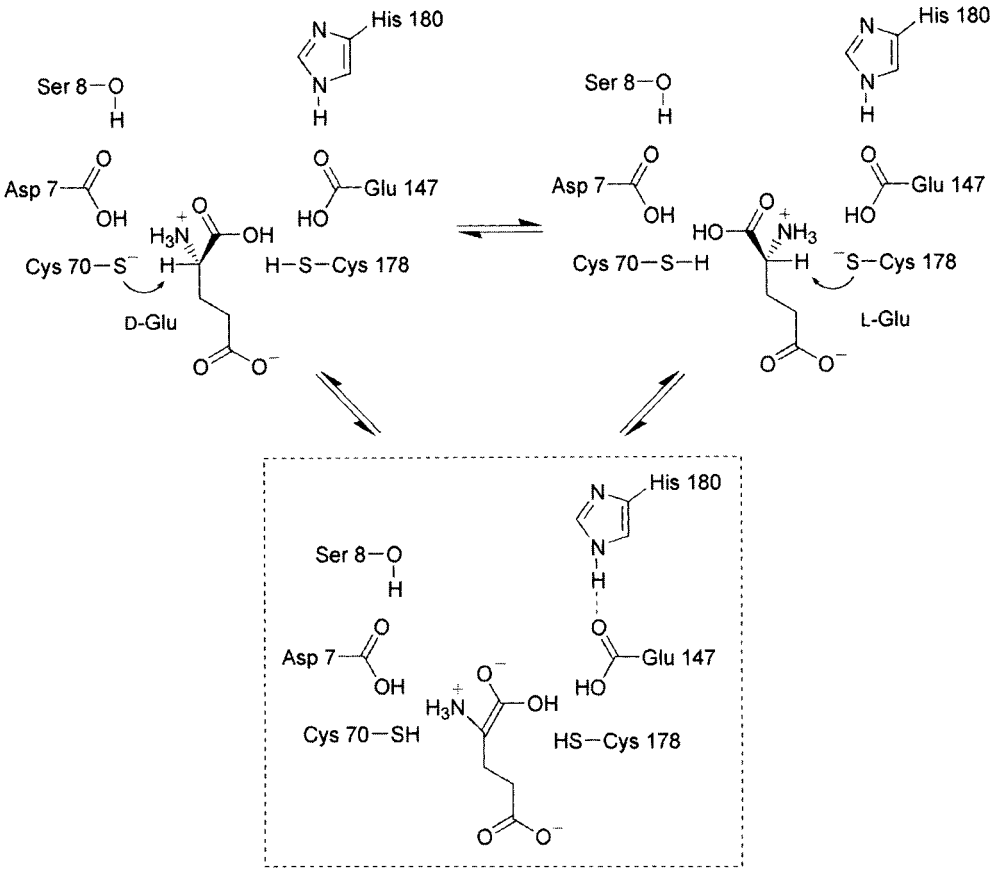


Figure 17-9. Mechanism of glutamate racemase reaction. Cys 70 and Cys178 serve as the bases to abstract an α-proton from the substrate, and a carbanion intermediate is formed. Alternatively, the racemization may proceed through a concerted mechanism. Reprinted from Hwang et al.[95].

toward D-N-hydroxyglutamate, whereas the C184S mutant was better than the wild-type. When L-*N*-hydroxyglutamate was used as a substrate, C73S was better but C184S was poorer than the wild-type. Thus, Glavas and Tanner concluded that Cys73 is responsible for the deprotonation of D-glutamate and Cys 184 is responsible for the deprotonation of L-glutamate[97].

17.2.2.1.4 Synthesis of D-Amino Acids with Glutamate Racemase

Nakajima et al.[98] have developed an efficient method for the synthesis of various D-amino acids from the corresponding α-keto acids and ammonia by coupling of four enzyme reactions catalyzed by D-amino acid aminotransferase[99], glutamate racemase[79, 91], glutamate dehydrogenase and formate dehydrogenase (Fig. 17-10). Various D-amino acids are produced by this method. Under the optimum conditions established by Nakajima et al.[98], D-enantiomers of valine, alanine, α-aminobutyrate,

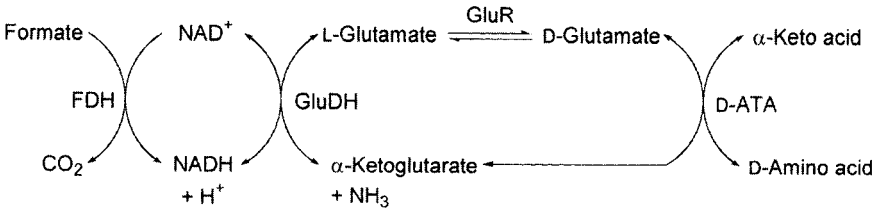


Figure 17-10. Enzymatic synthesis of D-amino acids by combination of glutamate racemase, glutamate dehydrogenase, D-amino acid aminotransferase and formate dehydrogenase reactions.

Table 17-4. Production of various D-amino acids by means of four purified enzymes: glutamate racemase, D-amino acid aminotransferase, glutamate dehydrogenase, and formate dehydrogenase[a].

D-Amino acids	Molar yield (%)
D-Valine	100
D-Alanine	100
D-α-Aminobutyrate	100
D-Aspartate	100
D-Leucine	84
D-Methionine	80
D-Serine	50
D-Histidine	36
D-Phenylalanine	28
D-Tyrosine	13

a Reprinted from N. Nakajima et al.[198].

leucine, methionine and aspartate are synthesized from their α-keto analogs with a molar yield higher than 80% under the conditions used (Table 17-4)[98]. D-Histidine and a few other D-amino acids, which are poor substrates of D-amino acid aminotransferase[99], are produced in a yield lower than 40% under the same conditions. However, Bae et al.[100] established an efficient method for production of D-phenylalanine and D-tyrosine by feeding α-keto acid intermittently in order to keep its concentration at less than 50 mM, above which the productivity decreased greatly (Fig. 17-11). By running the multi-enzyme system for 35 h, 48 g L^{-1} of D-phenylalanine and 60 g L^{-1} of D-tyrosine were produced with 100% of optical purity from the equimolar amounts of phenylpyruvate and hydroxyphenylpyruvate, respectively. An enzyme-membrane reactor system containing polyethyleneglycol-NAD^+ developed by Wandrey and associates[101] is probably applicable to this system. The production level of D-amino acids are mainly dependent on the stability of glutamate racemase. Therefore, thermostable glutamate racemases produced by *A. pyrophilus*[87] and *B. subtilis*[88] are probably useful as catalyst of this multi-enzyme system.

Yagasaki et al.[102] developed a new method for the synthesis of D-glutamate from L-glutamate by means of *E. coli* recombinant cells harboring a plasmid containing glutamate racemase gene from *L. brevis* ATCC 8287. L-Glutamate was first racemized to D,L-glutamate at pH 8.5, and L-glutamate was then decarboxylated at pH 4.2 by glutamate decarboxylase, which was inherently produced by the *E. coli* host cells.

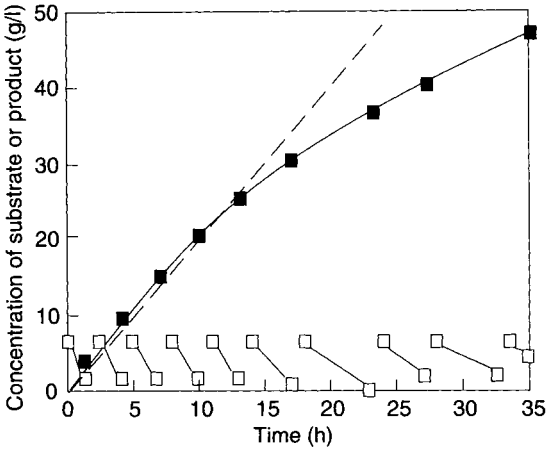


Figure 17-11. Production of D-phenylalanine by successive feeding of phenylpyruvate. Phenylpyruvate (□) was added intermittently. The dotted line indicates the expected productivity of D-phenylalanine (■) on the basis of the initial production rate. Reprinted from Bae et al.[100].

Starting from 100 g L^{-1} of L-glutamate, they obtained 50 g L^{-1} of D-glutamate in a 15 h reaction. D-Glutamate can be produced successively from L-glutamate with *L. brevis* ATCC 8287 cells because this strain produces both glutamate racemase and glutamate decarboxylase simultaneously. Thus, 50 g L^{-1} of optically pure D-glutamate was produced from 100 g L^{-1} of L-glutamate[103]. Oikawa et al.[104] replaced glutamate decarboxylase by glutamate oxidase because the oxidase has optimum pH values similar to that of glutamate racemase. They developed a bioreactor consisting of two columns sequentially connected and containing immobilized glutamate racemase from *B. subtilis* and L-glutamate oxidase from *Streptomyces* sp. X119–6: L-glutamate was racemized by the glutamate racemase column, and then L-glutamate was oxidized by the L-glutamate oxidase column. D-Glutamate was produced in about 90% of the theoretical yield[104].

17.2.2.2
Aspartate Racemase (E.C. 5.1.1.13)

D-Aspartate occurs in the peptidoglycan layer of bacterial cell walls, and is produced from L-aspartate through an aspartate racemase (E.C. 5.1.1.13) reaction[105]. The enzyme has been demonstrated as being present in various *Lactobacillus* and *Streptococcus* strains[106] such as *Lactobacillus fermenti*[105] and *Streptococcus faecalis*[107]. Recently, archaea such as *Desulfurococcus* strain SY[108] and *Thermococcus* strains[109] were shown to produce aspartate racemase. It is interesting to note that various other archaea such as *Pyrobaculum islandicum, Methanosarcina barkeri* and *Halobacterium salinarium* produce D-amino acids, although their function is not yet known[110].

Okada et al. purified the enzyme to homogeneity from the cell extract of *S. thermophilus*, the specific activity of the crude extract of which was elevated 3400-fold[106]. The gene encoding aspartate racemase was cloned from *S. thermophilus*, and overexpressed in *E. coli*[111]. The amount of the enzyme produced reached

about 20% of the total soluble proteins of the *E. coli* clone cells. Thus, the enzyme was efficiently purified to homogeneity from the clone cells[111]. The enzyme is a homodimer of a subunit with a molecular weight of about 28000. In addition to aspartate, cysteate and cysteine sulfinate are the only substrates of the enzyme: they are racemized at a rate of 88 and 51%, respectively, of that of L-aspartate[112]. The presence of the acidic group at the β-carbon is essential; none of asparagine, cysteine, serine, and alanine are the substrates. Both isomers of glutamate are also inert. The K_M values for L- and D-aspartate are 35 and 8.7 mM, respectively.

Aspartate racemase requires no cofactors and contains an essential cysteine residue in the same manner as glutamate racemase[80]. When L- or D-aspartate was incubated with aspartate racemase in tritiated water, tritium was incorporated preferentially into the product enantiomer. This is consistent with the results of glutamate racemase as described above[91].

Yamauchi et al.[112] concluded that aspartate racemase also uses two bases to remove and return the α-proton of the substrate. Aspartate racemase contains three cysteine residues: Cys 84, Cys 190 and Cys 197, and only Cys 84 is essential for the enzyme activity. The alkylation of one cysteine residue/dimer with 2-nitro-5-thiocyanobenzoic acid results in a complete loss of activity. Therefore, the enzyme shows a half-of-the-sites-reactivity[112]. Yamauchi et al.[112] suggested that the enzyme has a composite active site formed at the interface of two identical subunits in the same manner as proposed for proline racemase[92].

Kumagai and coworkers[113] developed an enzymatic procedure to produce D-alanine from fumarate by means of aspartase (E.C. 4.3.1.1), aspartate racemase, and D-amino acid aminotransferase (Fig. 17-12). Aspartase catalyzes conversion of fumarate into L-aspartate, which is racemized to form D-aspartate. D-Amino acid aminotransferase catalyzes transamination between D-aspartate and pyruvate to produce D-alanine and oxalacetate. This 2-oxo acid is easily decarboxylated spontaneously to form pyruvate in the presence of metals. Thus, the transamination proceeds exclusively toward the direction of D-alanine synthesis, and total conversion of fumarate into D-alanine was achieved.

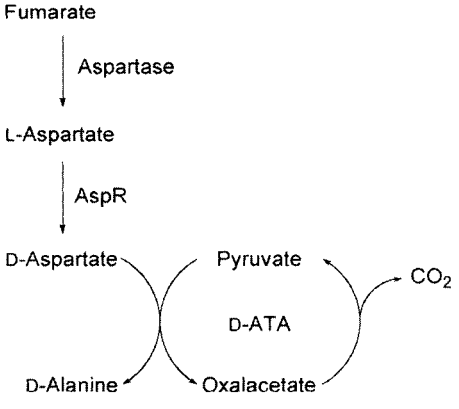


Figure 17-12. Enzymatic production of D-alanine by combination of aspartase, aspartate racemase, and D-amino acid aminotransferase reactions.

17.2.2.3
Diaminopimelate Epimerase (E.C. 5.1.1.7)

meso-α,ε-Diaminopimelate is the direct precursor of L-lysine, and is an essential component of the cell wall peptidoglycans in Gram negative bacteria. *meso*-α,ε-Diaminopimelate is formed from L-α,ε-diaminopimelate by diaminopimelate epimerase. The enzyme gene (*dap*F) was mapped at 85 min on the *E. coli* chromosome[114]. Richaud et al. isolated an *E. coli* mutant lacking diaminopimelate epimerase activity by insertional mutagenesis, and showed that the mutant does not require *meso*-α,ε-diaminopimelate in a minimal medium[114]. Thus, *meso*-α,ε-diaminopimelate epimerase encoded by the *dapF* gene is not essential for *E. coli*, but *meso*-α,ε-diaminopimelate still occurs in the mutant cells. Richaud et al. proposed that *E. coli* has another enzyme with diaminopimelate epimerase activity[114].

The diaminopimelate epimerase gene (*dap*F) was cloned from *E. coli*[114], and the amino acid sequence of the enzyme was deduced from the nucleotide sequence[115]. The enzyme was purified to homogeneity from the wild-type *E. coli* cells[93] and the recombinant *E. coli* cells carrying a plasmid coding for *dap*F gene[116]. The enzyme is composed of two identical subunits with a molecular weight of about 32 000. The enzyme is independent of PLP or of any other cofactors. The enzyme shows a V_{max} value of 132 μmol min^{-1} per mg of protein, and a K_M value of 0.24 mM for L-α,ε-diaminopimelate. The thiol group of Cys 73 of the enzyme is specifically labeled by a mechanism-based inactivator, 2-(4-amino-4-carboxybutyl)-2-aziridine carboxylic acid. Higgins et al. discovered an interesting similarity in amino acid sequences around the catalytically essential cysteine residue of proline racemase[92], hydroxyproline epimerase[94], and diaminopimelate epimerase (Cys 73), and proposed that PLP-independent racemases/epimerases derive from a common evolutionary origin[116]. However, no significant similarity in the entire amino acid sequence was found between diaminopimelate epimerase and glutamate racemase, and also between diaminopimelate epimerase and aspartate racemase.

Cirilli et al.[117] cloned the gene of diaminopimelate epimerase from *Haemophilus influenzae*, and purified and crystallized the enzyme. The enzyme is monomeric and has a unique protein fold, in which the amino terminal and carboxyl terminal halves of the molecule fold into structurally homologous and superimposable domains (Fig. 17-13). Cys 73 of the amino terminal domain is found in the disulfide linkage, at the domain interface, with Cys 217 of the carboxy terminal domain[117]. Thus, it is most conceivable that these two cysteine residues stay in reduced form in the active enzyme and function as the acid and base in the mechanism. Koo and Blanchard[118] explored a number of kinetic and isotope approaches to clarify the mechanism of the enzyme. However, which of the two cysteine residues is responsible for proton abstraction from the two enantiomeric Cα-H bonds is not yet known.

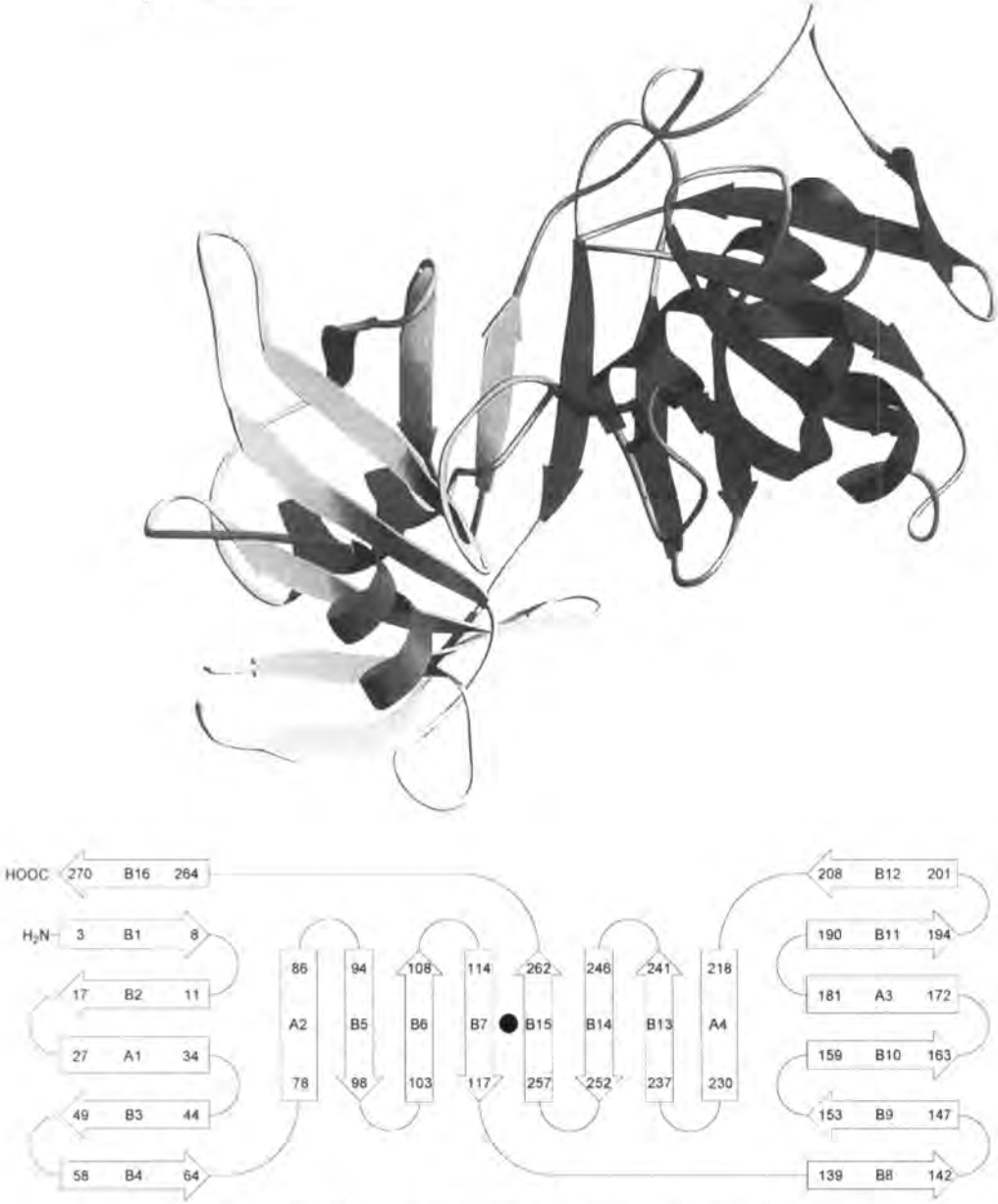


Figure 17-13. Top: Ribbon diagram of diaminopimelate epimerase from *Haemophilus influenzae*. The disulfide bridge between Cys 73 and Cys 217 connects domain I (residues 1–117 and 263–274) and domain II (residues 118–262). Bottom: Topology of the secondary structural elements of diaminopimelate epimerase. The position of pseudo-2-fold symmetry axis is indicated by the black dot between β-strands B7 and B8. Reprinted from M. Cirilli et al.[117].

17.2.2.4
Proline Racemase (E.C. 5.1.1.4)

Proline racemase occurs in *Clostridium sticklandii*, which produces δ-aminovalerate from L-proline. Proline racemase and D-proline reductase are responsible for the conversion: L-proline is racemized by proline racemase to form D-proline, which is converted into δ-aminovalerate by D-proline reductase (E.C. 1.4.4.1).

Rudnick and Abeles purified proline racemase to 95% homogeneity from *Clostridium sticklandii*, and characterized it[92]. The enzyme is composed of two identical subunits with a molecular weight of about 38000, and is independent of any cofactors or metals. Most amino acid racemases require pyridoxal 5'-phosphate, which labilizes the bond between the α-hydrogen and the chiral center by aldimine formation with the α-amino group of the substrate. However, PLP is not involved in the reaction of proline racemase acting on an α-imino acid. The enzyme also acts on 2-hydroxy-L-proline and 2-allo-hydroxy-D-proline although slowly: they are epimerized at a rate of 2 and 5% of the rate of L-proline racemization, respectively. L-Proline and D-proline showed K_M values of 2.9 and 2.5 mM, respectively[119].

Pyrrole-2-carboxylate is a competitive inhibitor of proline racemase, and stoichiometrically binds with the enzyme (1 mol per dimer). Thiol groups of the enzyme are alkylated by iodoacetate at a stoichiometry of 1 mol of cysteine residue per mol subunit. However, the enzyme is inactivated completely by modification of only one cysteine residue per dimer. Thus, Rudnick and Abeles proposed a reaction scheme in which the active site is located at the interface of two identical subunits, each of which furnishes one of the two active site thiol groups positioning appropriately at the composit active site: a thiolate anion derived from one thiol group abstracts the α-proton from the substrate, and another thiol group protonates the intermediate derived from the substrate from the opposite face[92]. They proposed occurrence of two forms of free proline racemase: one binds with D-proline and the other binds with L-proline. According to their proposed mechanism, the product enantiomer is released much faster than the release of the substrate-derived proton. The proton release also proceeds much faster than the interconversion of the two forms of the enzyme. Knowles and coworkers defined the energetics and delineated the complete free energy profile for the proline racemase reaction[119–125].

Yagasaki and Ozaki[126] developed a method for production of D-proline from L-proline using the recombinant proline racemase of *Clostridium sticklandii*. L-Proline was degraded by *Candida* sp. PRD-234, and optically pure D-proline was obtained.

17.2.3
Other Racemases and Epimerases Acting on Amino Acid Derivatives

17.2.3.1
2-Amino-Δ2-thiazoline-4-carboxylate Racemase

Sano et al.[127] have found several bacterial strains that are capable of producing L-cysteine from D,L-2-amino-2-thiazoline-4-carboxylate (ATC), an intermediate in the

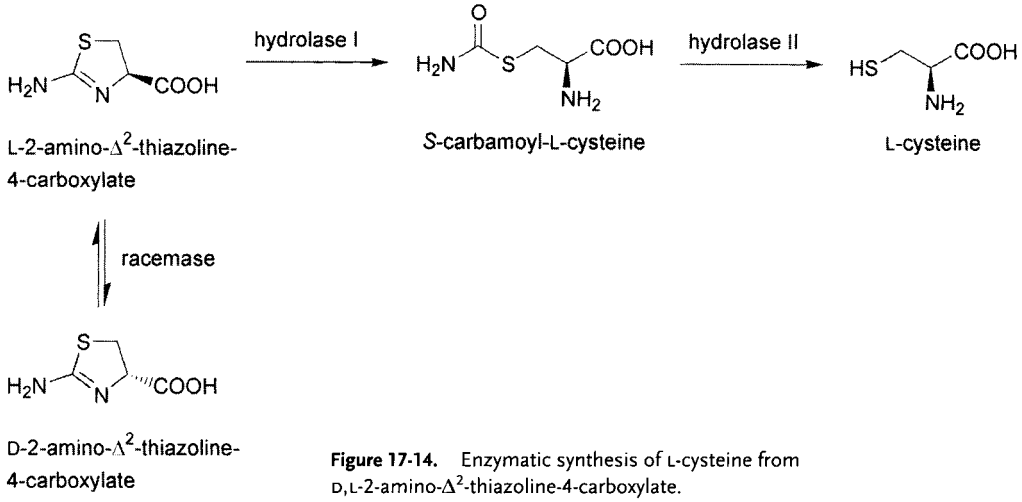


Figure 17-14. Enzymatic synthesis of L-cysteine from D,L-2-amino-Δ^2-thiazoline-4-carboxylate.

chemical synthesis of D,L-cysteine. These include several *Pseudomonas* species isolated from soil and other strains belonging to different genera such as *E. coli*, *Bacillus brevis*, and *Micrococcus sodenensis*[127]. Three enzymes are probably involved in this pathway: L-ATC hydrolase, *S*-carbamoyl-L-cysteine hydrolase and ATC racemase (Fig. 17-14). *Pseudomonas thiazolinophilum* isolated from soil was shown to have the highest activity of the enzymes that produce L-cysteine from D,L-ATC. The enzymes are inducibly formed in the bacterial cells by addition of D,L-ATC to the growth medium.

Degradation of L-cysteine by cysteine desulfhydrase or other PLP enzymes present in the cells was successfully prevented by addition of hydroxylamine or semicarbazide to the incubation mixture. A mutant strain of *Ps. thiazolinophilum* lacking cysteine desulfhydrase was isolated and used to produce L-cysteine from D,L-ATC in a molar yield of 95% and at a product concentration of 31.4 g L^{-1}[128]. *Pseudomonas desmolytica* AJ 3872, one of the L-cysteine producers isolated was found to lack the ability to convert D-ATC into L-cysteine: it is an ATC racemase-deficient strain[129]. However, little is known about the enzymological properties and function of the racemase.

Among the three enzymes participating in L-cysteine production, L-ATC hydrolase was found to be the least stable[130]. However, the stability of L-ATC hydrolase was sharply enhanced as water activity decreased from 0.93 to 0.80. In the absence of sorbitol, the stability of L-ATC hydrolase increased in proportion to ionic strength. Thus, Ryu et al. succeeded in enhancing the half life of L-ATC hydrolase by 10-fold to 20-fold in sorbitol-salt mixtures[130].

17.2.3.2
Hydantoin Racemase

5-Substituted hydantoin derivatives have been used as precursors for D- and L-amino acids in chemical synthesis. However, they are hydrolyzed enantioselectively by the enzymes named hydantoinases: some act specifically on D-5-substituted hydantoins, and others on the L-isomers. *N*-Carbamoyl amino acids formed are also hydrolyzed enantiospecifically by *N*-carbamoyl amino acid amidohydrolases to produce D- or L-amino acids (Fig. 17-15). Since the Kanegafuchi Chemical Industry, Japan, commercialized an enzymatic procedure for the production of D-*p*-hydroxyphenylglycine, which is a building block for the semisynthetic β-lactam antibiotic amoxycillin, various processes for amino acid production by means of hydantoinases have been developed[131–133].

Subsequent to the discovery that hydantoin is hydrolyzed by extracts of mammalian livers[134] and plant seeds[135], various microorganisms have been shown to utilize D- and L-5-substituted hydantoins as a sole carbon or nitrogen source by means of D- as well as L-specific hydantoinases inducibly formed[131–133].

Distribution of D-hydantoinase in microorganisms has been shown by Yamada and coworkers[136]. The enzyme is identical to dihydropyrimidinase (E.C. 3.5.2.2), and is widely distributed in bacteria, in particular in *Klebsiella*, *Corynebacterium*, *Agrobacterium*, *Pseudomonas*, and *Bacillus*, and also in actinomycetes such as *Streptomyces* and *Actinoplanes*. The enzyme activity occurs also in eukaryotes: yeasts, molds, plants and mammals. *Pseudomonas putida* was found to be the best strain, which produced D-hydantoinase most abundantly and inducibly by addition of 5-methylhydantoin. Most of D-hydantoinase producers form *N*-carbamoyl D-amino acids from the corresponding 5-substituted hydantoins. Accordingly, to obtain free D-amino acids, *N*-carbamoyl amino acids need to be isolated and hydrolyzed chemically or enzymatically. However, a few bacterial strains produce *N*-carbamoyl D-amino acid amidohydrolase in addition to D-hydantoinase. Thus, optically pure D-amino acids were produced from D-hydantoins with these bacterial cells. Olivieri et al.[137] found that *Agrobacterium tumefaciens* cells grown on uracil as a sole nitrogen source catalyze the complete conversion of racemic hydantoins into D-amino acids. Hartley et al.[138] obtained a mutant strain which expresses both the hydantoinase and *N*-carbamoylamino acid amidohydrolase in the absence of an inducer. In contrast, other bacterial strains belonging to the genera of *Flavobacterium*[139], *Arthro-*

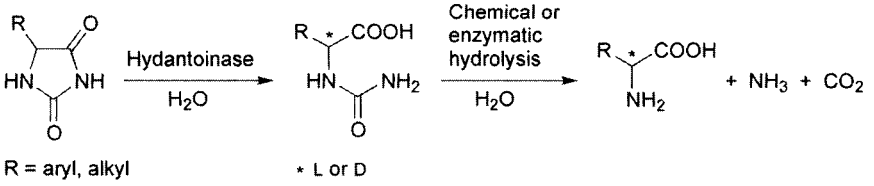


Figure 17-15. Enzymatic synthesis of D- or L-amino acids from 5-substituted D,L-hydantoins through *N*-carbamoyl-D- or L-amino acids.

bacter[140], *Pseudomonas*[141, 142], and *Bacillus*[143–145] convert whole racemic 5-substituted hydantoins into the corresponding L-amino acids. In these bacteria, 5-substituted hydantoins are hydrolyzed by L-hydantoinase to form *N*-carbamoyl L-amino acids, which are hydrolyzed further to L-amino acids by *N*-carbamoyl L-amino acid amidohydrolase in the same manner as described above except that the enzymes involved show opposite stereospecificity. 5-Mono-substituted hydantoins can racemize spontaneously under weakly alkaline conditions, and this chemical racemization participates at least partly in the total conversion of the racemic hydantoins into free L- or D-amino acids. However, if chemical racemization proceeds only slowly[146], a hydantoin racemase was suggested to occur and participate in the total conversion[146, 147].

Watabe et al.[148] isolated a plasmid which is responsible for the conversion of 5-substituted hydantoins into the corresponding L-amino acids from a soil bacterium, *Pseudomonas* sp. NS 671, which is able to convert racemic 5-substituted hydantoins into the corresponding L-amino acids. The genes involved in the conversion were cloned from the *Pseudomonas* plasmid into *E. coli*, and functions of four genes were identified and named *hyu*A, *hyu*B, *hyu*C and *hyu*E. Both *hyu*A and *hyu*B are required for the conversion of D- and L-5-substituted hydantoins into the corresponding *N*-carbamoyl-D- and *N*-carbamoyl-L-amino acids, respectively, although the individual reactions catalyzed by the gene products have not yet been identified. *Hyu*C codes for an *N*-carbamoyl-L-amino acid amidohydrolase, while *hyu*E is a hydantoin racemase gene[149]. Significant nucleotide sequence similarity was found between *hyu*A and *hyu*C (43%), and also between *hyu*B and *hyu*C (46%). Watabe et al. suggested that these genes have evolved from a common ancestor by gene duplication[148]. However, no proteins registered in NBRF and SWISS protein data bases showed similarity with the deduced amino acid sequences of the four genes.

Wagner and associates purified hydantoin racemase from *Arthrobacter aurescens* DSM 3747 and characterized it[133]. Watabe et al.[149] also purified the enzyme from *E. coli* clone cells harboring a plasmid coding for the enzyme gene derived from *Pseudomonas* sp. NS 671. The *Pseudomonas* enzyme is a hexamer composed of a subunit with a molecular weight of about 32 000, which is consistent with the value deduced from the amino acid sequence. The D- and L-isomers of 5-(2-methylthioethyl)hydantoin and 5-isobutyrylhydantoin are racemized effectively. D-5-(2-Methylthioethyl)hydantoin is racemized at a V_{max} value (79 μmol min^{-1} mg^{-1}) which is about 2.5 times higher than that for the L-isomer. Wiese et al.[150] cloned the hydantoin racemase gene from *Arthrobacter aurescens* DSM 3747 and purified the enzyme to homogeneity. The *Arthrobacter* enzyme has a molecular mass of 25.1 kDa[150] and acts on aromatic and aliphatic hydantoin derivatives such as 5-indolylmethylhydantoin, 5-benzylhydantoin, 5-(*p*-hydroxybenzyl)hydantoin, 5-(2-methylthioethyl)hydantoin, and 5-isobutylhydantoin[133], although hydantoins with arylalkyl side chains are preferred substrates[159]. Free amino acids, amino acid esters and amides are inert, but the enzyme suffers from inhibition by aliphatic substrates such as L-5-methylthioethylhydantoin. The hydrogen at the chiral center of a substrate, D-5-indolylmethylenehydantoin, is exchanged with solvent deuterium

during racemization[150]. Pietzsch et al.[152] established a method for the synthesis of optically pure D-3-trimethylsilylalanine from D,L-5-trimethylsilylmethylhydantoin in 88% yield and 95% enantiomeric excess with whole resting cells of *Agrobacterium* sp. IP I 671, immobilized in a Ca-alginate matrix. On the other hand, L-3-trimethylsilylalanine was also prepared from the racemic substrate by enantiomer-specific hydrolysis of the L-form in the presence of L-*N*-carbamoylase from *Arthrobacter aurescens* DSM 3747[152].

Watabe et al. found that the *Pseudomonas* enzyme is inactivated by a substrate, L-5-methylhydantoin, during racemization[151]. However, the enzyme was not affected by the D-isomer. Both enantiomers of 5-isopropylhydantoin inactivated the enzyme to the same extent. Interestingly, divalent sulfur-containing compounds such as methionine, cysteine, glutathione, and biotin protected the enzyme effectively from inactivation. *E. coli* cells expressing the racemase are capable of racemizing all of these hydantoin derivatives: the enzyme is protected from inactivation by divalent sulfur compounds occurring in the cells. Watabe et al. concluded that the protective effect by the divalent sulfur-compounds is not due to their reducing activity[151]. Both *Pseudomonas*[151] and *Arthrobacter*[133] enzymes are inhibited strongly by Cu^{2+}. The *Arthrobacter* enzyme is completely inhibited by $HgCl_2$ and iodoacetamide, and stimulated by addition of dithiothreitol[150]. Therefore, the enzyme may contain essential cysteine residues, which are possibly modified by some activated intermediate derived from the particular substrates leading to the enzyme inactivation.

E. coli cells carrying a plasmid coding for *hyu*A, *hyu*B, *hyu*C, and *hyu*E convert only D-5-(2-methylthioethyl)hydantoin into L-methionine. On the other hand, *E. coli* cells harboring a plasmid coding for only *hyu*A, *hyu*B, and *hyu*C first convert the L-hydantoin, then the D-isomer is hydrolyzed slowly when the L-isomer is depleted. Therefore, Watabe et al. believe that D-5-(2-methylthioethyl)hydantoin is only converted into L-methionine in the presence of the hydantoin racemase[151]. The mechanism of stereospecific conversion of D,L-5-substituted hydantoins to the corresponding L-amino acids by *Pseudomonas* sp. strain NS 671 has been clarified by Ishikawa et al.[153]: D,L-5-substituted hydantoins are converted exclusively into the L-forms of the corresponding *N*-carbamoylamino acids by the hydantoinase in combination with hydantoin racemase, and then the *N*-carbamoyl-L-amino acids are converted into L-amino acids by *N*-carbamoyl-L-amino acid amidohydrolase (Fig. 17-16).

By directed evolution May et al.[154] succeeded in inverting the enantioselectivity of D-hydantoinase from *Arthrobacter* sp. DSM 9771 into an L-selective enzyme. The improved hydantoinase also acquired a five-fold increase in activity. The recombinant *E. coli* cells expressing three heterologous genes (i.e. the evolved L-hydantoinase, L-*N*-carbamoylase, and hydantoin racemase) were found to produce 91 mM L-methionine from 100 mM D-5-(2-methylthioethyl)hydantoin in less than 2 h[154].

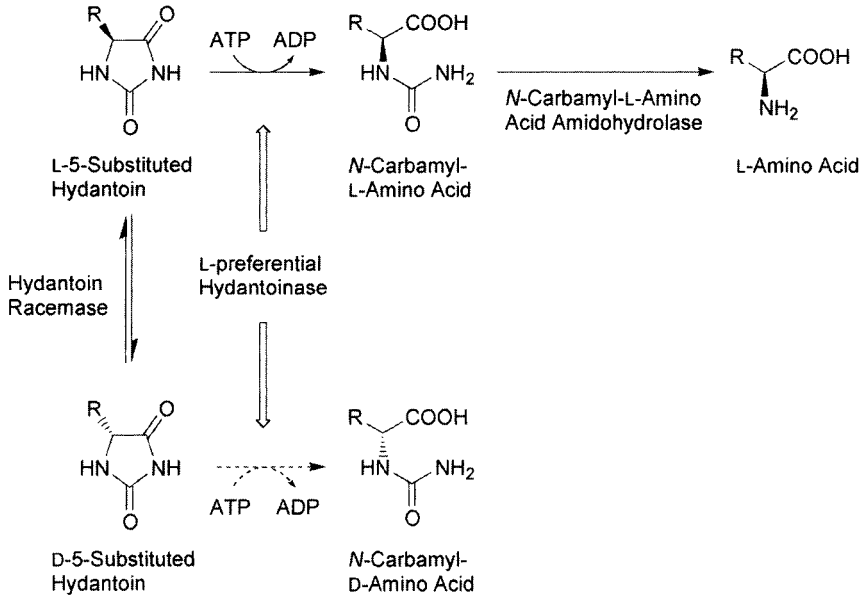


Figure 17-16. Stereospecific conversion of D,L-5-substituted hydantoins into the corresponding L-amino acids by *Pseudomonas* sp. NS 671. Reprinted from Ishikawa et al.[153].

17.2.3.3
N-Acylamino Acid Racemase

L-Aminoacylases (E.C. 3.5.1.14) catalyze the hydrolysis of the amide bond of various *N*-acyl-L-amino acids, such as *N*-acetyl-, *N*-chloroacetyl- and *N*-propionyl-L-amino acids[155], and is widely distributed in animals[155–157], plants[158, 159], and microorganisms[160, 161]. Greenstein[155] first studied the reactivity of pig kidney enzyme, and showed its application to the optical resolution of racemic amino acids. Chibata et al.[162] found that L-aminoacylase is produced abundantly by fungal species belonging to the genera *Aspergillus* and *Penicillium*. L-Aminoacylases were purified from pig kidney and *A. oryzae*, and their reaction mechanism and physiological function were studied[160, 163–165]. Cho et al.[166] showed that various thermophilic *Bacillus* strains produce thermostable L-aminoacylase, and purified it to homogeneity from *Bacillus thermoglucosidius* DSM 2542, which produces the enzyme most abundantly. L-Aminoacylases of pig kidney, *Aspergillus oryzae* and *B. thermoglucosidius* share many features with each other: they contain Zn^{2+} as a prosthetic metal, are strongly activated by Co^{2+}, and have a pH optimum in the range of 8.0–8.5.

Sugie and Suzuki[167] demonstrated the occurrence of D-aminoacylase, which specifically hydrolyzes the amide bond of *N*-acyl-D-amino acids, in actinomycetes, and applied the enzyme to the production of D-phenylglycine. Recently, a new D-aminopeptidase was found in *Alcaligenes denitrificans*, and shown to act on various *N*-acyl-D-amino acids including *N*-acetyl-D-methionine[168, 169].

N-Acylamino acids are usually racemized much more readily than the corresponding free amino acids. Therefore, by combination of chemical racemization and enantioselective hydrolysis of *N*-acylamino acids, racemates of *N*-acylamino acids can be fully converted into the desired enantiomer of the free amino acids according to the stereospecificity of the aminoacylases used. For example, L-tryptophan is produced industrially by combination of chemical racemization of *N*-acetyltryptophan and enantiospecific hydrolysis of its L-isomer with the *Aspergillus* L-aminoacylase, which shows high reactivity towards *N*-acyl derivatives of aromatic L-amino acids. When *N*-acetyl-D,L-tryptophan is incubated with the fungal enzyme, *N*-acetyl-L-tryptophan is selectively hydrolyzed to L-tryptophan, which is then crystallized from the solution. *N*-Acetyl-D-tryptophan in the mother liquor is racemized with acetic anhydride, and the racemate is again used as a starting material. In principle, D- and L-amino acids can be produced from their corresponding *N*-acyl derivatives in the same manner, provided that *N*-acyl derivatives of the desired amino acids serve as the substrates of the available aminoacylases, and are racemized chemically without any major loss by decomposition. However, the chemical racemization can be achieved only under extreme conditions in order for the aminoacylases to be inactivated, and the enzymes are usually required to be saved for the subsequent cycles for reasons of economy. Therefore, the antipode of the substrate is separated from the enzyme and preferably from the product in order to avoid its possible racemization. Tosa et al. have developed a continuous method to produce L-tryptophan, which is now utilized in industry, by means of the *Aspergillus* L-aminoacylase immobilized on DEAE-Sephadex[170].

Takahashi and Hatano of Takeda Chemical Industries, Japan, succeeded in finding a racemase that acts on *N*-acylamino acids, but not on the corresponding free amino acids, and named it acylamino acid racemase[171]. They have established a method of producing optically active α-amino acids from the corresponding D,L-*N*-acylamino acids by means of the acylamino acid racemase and aminoacylases.

Acylamino acid racemase occurs widely in various actinomycete strains belonging to the genera of *Streptomyces*, *Actinomadura*, *Actinomyces*, *Jensenia*, and *Amycolatopsis*[172]. The enzyme was purified to homogeneity from *Streptomyces atratus* Y-53, which shows the highest enzyme activity among the strains tested[173]. The enzyme is composed of 6 subunits with identical molecular masses (about 41000), and shows a molecular mass of 244000 in the native state. Tokuyama and Hatano[174] purified thermostable *N*-acylamino acid racemase from *Amycolatopsis* sp. TS-1–60 and purified it to homogeneity. The molecular masses of the native enzyme and the subunit are 300000 and 40000, respectively. The enzyme is stable at 55 °C for 30 min. The enzyme catalyzes the racemization of *N*-acylamino acids such as *N*-acetyl-L- or D-methionine, *N*-acetyl-L-valine, *N*-acetyl-L-tyrosine and *N*-chloroacetyl-L-valine (Table 17-5). In addition, the enzyme also catalyzes racemization of dipeptide L-alanyl-L-methionine. By contrast, *N*-alkylamino acids and methyl and ethyl esters of *N*-acetyl-D- and L-methionine are not racemized. The apparent K_M values for *N*-acetyl-L-methionine and *N*-acetyl-D-methionine are 18.5 mM and 11.3 mM, respectively. The enzyme activity is markedly enhanced by the addition of divalent metal ions such as Co^{2+}, Mn^{2+} and Fe^{2+} and inhibited by addition of EDTA and *p*-

Table 17-5. Substrate specificity of acylamino acid racemase[a].

Substrate	Relative activity
N-Acetyl-D-methionine	100
N-Acetyl-L-methionine	100
N-Formyl-D-methionine	40
N-Formyl-L-methionine	63
N-Acetyl-D-alanine	33
N-Acetyl-L-alanine	21
N-Benzoyl-D-alanine	14
N-Acetyl-D-leucine	37
N-Acetyl-L-leucine	74
N-Acetyl-D-phenylalanine	64
N-Acetyl-L-phenylalanine	84
N-Chloroacetyl-D-phenylalanine	90
N-Chloroacetyl-L-phenylalanine	112
N-Acetyl-D-tryptophan	10
N-Acetyl-L-tryptophan	8
N-Acetyl-D-valine	35
N-Acetyl-L-valine	19
N-Chloroacetyl-D-valine	80
N-Chloroacetyl-L-valine	105
N-Acetyl-D-allo isoleucine	33

a Inert: D- and L-methionine, D- and L-alanine, D- and L-leucine, D-and L-phenylalanine, D- and L-tryptophan, D- and L-valine.

chloromercuribenzoate. The gene of *N*-acylamino acid racemase was cloned from *Amycolatopsis* sp. TS-1–60[175], and overexpressed in *E. coli* host cells with T7 promoter[176]. The gene codes for a protein of 368 amino acids with a molecular mass of 39411 Da. Palmer et al.[177] found that *N*-acylamino acid racemase of *Amycolaptosis* sp. TS-1–60 is similar to an unidentified protein encoded by the *Bacillus subtilis* genome. *N*-Acylamino acid racemase efficiently catalyzes an *O*-succinylbenzoate synthase reaction, which is responsible for menaquinone biosynthesis.

Tokuyama et al.[172] found that most of acylamino acid racemase-producing strains produce not only acylamino acid racemase but also aminoacylases; one of either D- or L-aminoacylase or both of them. Moreover, acylamino acid racemase shows the optimum pH at around 8.0, which is close to that of aminoacylases. Therefore, *N*-acylamino acid can be converted as a whole into L- or D-amino acids in one step by means of microbial cells of appropriate strains producing either L- or D-aminoacylase in addition to acylamino acid racemase.

17.2.3.4
Isopenicillin N Epimerase

Isopenicillin N is a precursor of penicillin, and synthesized from δ-(L-aminoadipoyl)-L-cysteinyl-D-valine by isopenicillin N synthetase[178]. Isopenicillin N is then converted into penicillin N by isopenicillin N epimerase. Penicillin N is ring-expanded to deacetoxycepharosporin C by penicillin N expandase. The latter compound is

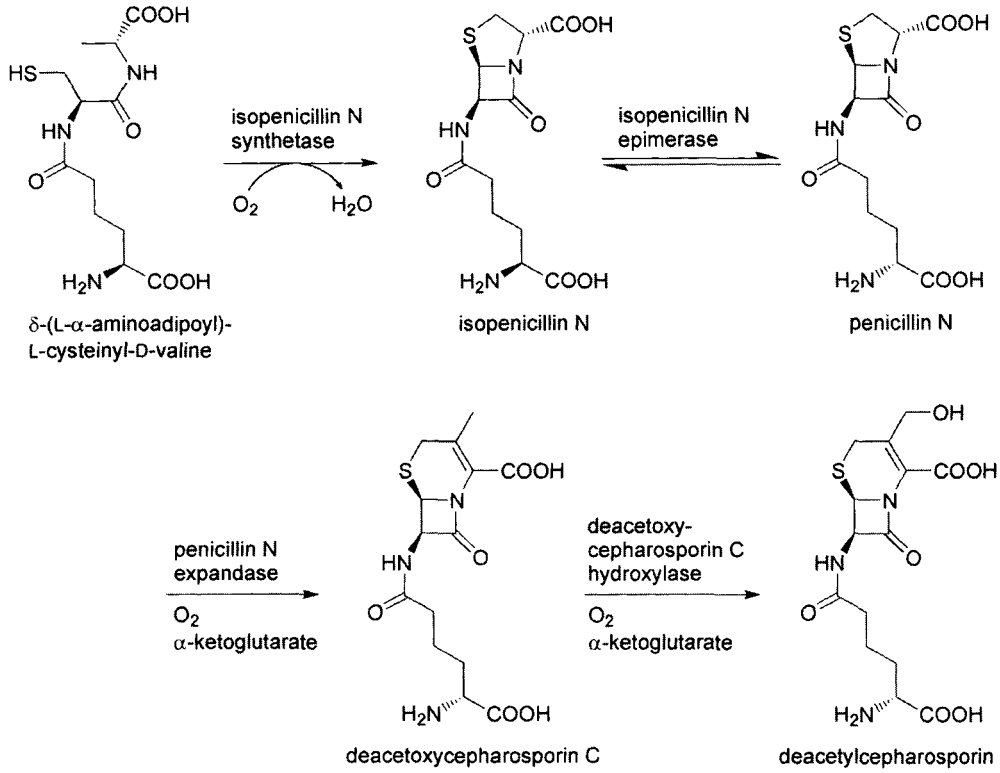


Figure 17-17. Biosynthetic pathway for cepharosporin C.

hydroxylated to form deacetylcepharosporin C by deacetoxycepharosporin C hydroxylase. These reactions proceed sequentially in the biosynthesis of cepharosporin C in *Streptomyces clavuligerus*, a producer of various β-lactam antibiotics[179, 180] (Fig. 17-17). However, in *Cepharosporium acremonium*, conversion of penicillin N into deacetoxycepharosporin C is catalyzed by a bifunctional enzyme, penicillin N expandase/deacetoxycepharosporin C hydroxylase in *Cepharosporium acremonium*[181].

Isopenicillin N epimerase activity, demonstrated in the extract of *Cepharosporium acremonium* protoplasts was found to be very unstable[182]. Usui and Yu[183], however, succeeded in purifying the enzyme to homogeneity after development of a simple assay procedure of the enzyme. They studied its enzymological properties[183]. The enzyme has a monomeric structure with a molecular mass of 47 000. The enzyme contains 1 mol of PLP per mol of protein. The enzyme shows a V_{max} value of 3.93 μmol min^{-1} per mg and a K_M of 0.30 mM for isopenicillin N, whereas it shows a V_{max} of 9.47 μmol min^{-1} per mg and a K_M of 0.78 mM for penicillin N. The K_{eq} value for the conversion between isopenicillin N and penicillin N is 1.09, which is in good agreement with the theoretical value. In addition to isopenicillin N and penicillin N, deacetoxycepharosporin C was epimerized only slowly: the rate relative

to isopenicillin N is about 1 %. However, the following penicillin derivatives are inert: deacetylcepharosporin C, ceparosporin C, δ-(L-α-aminoadiopoyl)-L-cysteinyl-D-valine, L-α-aminoadipate, and D-α-aminoadipate. The enzyme is inhibited strongly by thiol reagents such as *p*-chloromercuribenzoate[183].

17.2.4
Racemization and Epimerization at Hydroxyl Carbons

Various epimerases acting on carbohydrate derivatives and acyl-CoA derivatives were demonstrated, purified, and characterized as reviewed previously[184]. Lactate racemase (E.C. 5.1.2.1) is the first racemase to he discovered[1–4]. The mechanism of lactate racemase reaction was studied with the enzyme preparations partially purified from *Clostridium butyricum*[185]. Hiyama et al.[186] highly purified the enzyme from *Lactobacillus sake*, but little is known about its enzymological properties. In contrast, mandelate racemase (E.C. 5.1.2.2) is the enzyme best characterized among various racemases and epimerases: its tertiary structure and functional groups that participate directly in catalysis has been clarified.

17.2.4.1
Mandelate Racemase (E.C. 5.1.2.2)

Mandelate racemase catalyzes the racemization of mandelate, which is the first step of the mandelate assimilation pathway in *Pseudomonas putida*. Although the mandelate pathway occurs widely in various bacteria, fungi and yeasts, most of them utilize one enantiomer or the other of mandelate in a benzoate-forming pathway. A few strains such as *Acinetobacter calcoaceticus*[187] and *Aspergillus nigar*[188] are capable of using both enantiomers with two complementary dehydrogenases with different stereospecificities. However, a single strain of *Pseudomonas putida* producing mandelate racemase can utilize both enantiomers[189].

In *Pseudomonas putida*, D-mandelate is converted into L-mandelate by mandelate racemase, then oxidized to benzoylformate by mandelate dehydrogenase (Fig. 17-18). Benzoylformate decarboxylase is the second enzyme of the pathway and catalyzes decarboxylation of benzoylformate to form benzaldehyde, which is oxidized to benzoate by NAD- and NADP-linked benzaldehyde dehydrogenases. The genes encoding these five enzymes constitute an operon that is induced by either enantiomer of mandelate[190]. Stecher et al.[191] established large-scale production of mandelate racemase by *Pseudomonas putida* ATCC12633 by optimization of enzyme induction: both glucose and mandelate were added to the culture right from the start as the carbon source. Thus, about 300-fold enhancement in the enzyme production was achieved. Strauss et al.[192] showed that immobilized mandelate racemase is an efficient biocatalyst used for repeated batch reactions to produce (*R*)-mandelate from (*S*)-mandelate under mild conditions.

Kenyon and coworkers purified mandelate racemase to homogeneity, and characterized it[189]. Divalent metal ions such as Mg^{2+}, Mn^{2+}, Co^{2+}, and Ni^{2+} were required for the catalysis. In addition to mandelate, *p*-hydroxymandelate and *p*-(bromome-

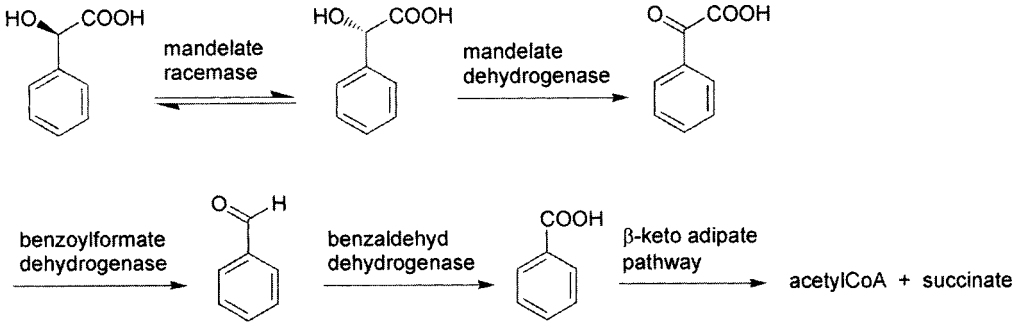


Figure 17-18. Mandelate assimilation pathway in *Pseudomonas putida*.

thyl)mandelate serve as the substrates. *p*-(Bromomethyl)mandelate is decomposed to *p*-(methyl)benzoylformate and bromide by action of the enzyme. The K_M values for D- and L-mandelate are 0.23 and 0.26 mM, respectively.

Ransom et al.[193] cloned the gene for mandelate racemase from *Pseudomonas putida* in *Pseudomonas aeruginosa* on the basis of the inability of the latter strain to grow on D-mandelate as a sole carbon source. The amino acid sequence was deduced from the nucleotide sequence, and the predicted molecular mass of the enzyme was 38750[193]. The enzyme is composed of eight identical subunits. The crystal structure of mandelate racemase has been solved and refined at 2.5 Å resolution[194]. The secondary, tertiary and quaternary structures of mandelate racemase are quite similar to those of muconate lactonizing enzyme[195, 196]. Mandelate racemase is composed of two major structural domains and a small C-terminal domain. The N-terminal domain has an α + β structure, and the central domain has an α/β-barrel topology. The C-terminal domain consists of an L-shaped loop.

Divalent metal ions, which are essential catalytically, are ligated by three distal carboxyl groups of Asp 195, Glu 221, and Glu 247, all of which occur at the central domain[194]. The active site location was determined by analysis of a complex between mandelate racemase and *p*-iodomandelate, whose iodine atom has high electron density and contributes greatly to the analysis. The active site of the enzyme is located between the two major domains. The ionizable groups of Lys 166 and His 297 are located at the positions interacting with the chiral center of the substrate (Fig. 17-19). Neidhart et al.[194] proposed that they participate in general acid/base catalysis: Lys 166 abstracts the α-proton of L-mandelate, and His 297 abstracts the α-proton from D-mandelate. Landro et al.[197] then replaced His 297 by asparagine, analyzed the crystal structure of the H297N mutant enzyme at 2.2 Å resolution, and studied the mechanism of catalysis of the mutant enzyme. Although the mutant enzyme has no mandelate racemase activity, it catalyzes the stereospecific elimination of bromide from *p*-(bromomethyl)-L-mandelate at a rate equivalent to that catalyzed by the wild-type enzyme. Moreover, the mutant enzyme catalyzes exchange of the α-hydrogen of L- but not D-mandelate with deuterium in deuterium oxide at a rate 3.3 times less than that of the wild-type enzyme. Thus, Landro et al.[197, 198] concluded that the mandelate racemase reaction proceeds through a two-base

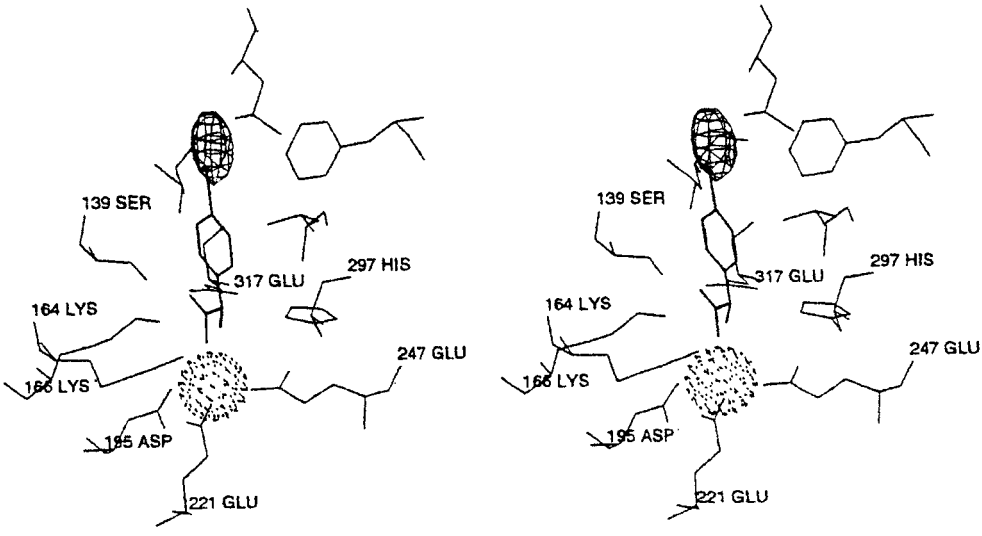


Figure 17-19. Models of the mandelate racemase active site with complexed substrate, *p*-iodomandelate. Reprinted from Neidhart et al.[194].

mechanism in which Lys 166 abstracts the α-proton from L-mandelate and His 297 abstracts the α-proton from D-mandelate (Fig. 17-20). In fact, the X-ray crystal studies of mandelate racemase inactivated by (*R*)-α-phenylglycidate revealed that the ε-amino group of Lys 166 is covalently bound to the distal carbon of the epoxide ring[199]. K166R mutant enzyme catalyzes the stereospecific elimination of bromide ion from (*R*)-*p*-(bromomethyl)mandelate to form *p*-(methyl)benzoylformate at a rate similar to that catalyzed by the wild-type enzyme[200], while H297N acts stereospecifically on (*S*)-*p*-(bromomethyl)mandelate[201]. This is compatible with the mechanism that Lys 166 and His 297 participate as the (*S*)- and (*R*)-specific catalyst, respectively. Bearne and Wolfenden[202] proposed that the complementary nature of the structures of mandelate racemase and its substrate is optimized in the transition state otherwise the general acid-general base catalysis will not become an efficient mode of catalysis.

17.3 Isomerizations

We describe here the enzymological characteristics and application of isomerases, especially D-xylose (glucose) isomerase, phosphoglucose isomerase, triose phosphate isomerase, L-rhamnose isomerase, L-fucose isomerase, maleate *cis-trans* isomerase, and unsaturated fatty acid *cis-trans* isomerase. *N*-Acetyl-D-glucosamine 2-epimerase is not an isomerase, but for convenience we will also describe the characteristics and use of the enzyme because this section deals with sugar-metabolizing enzymes.

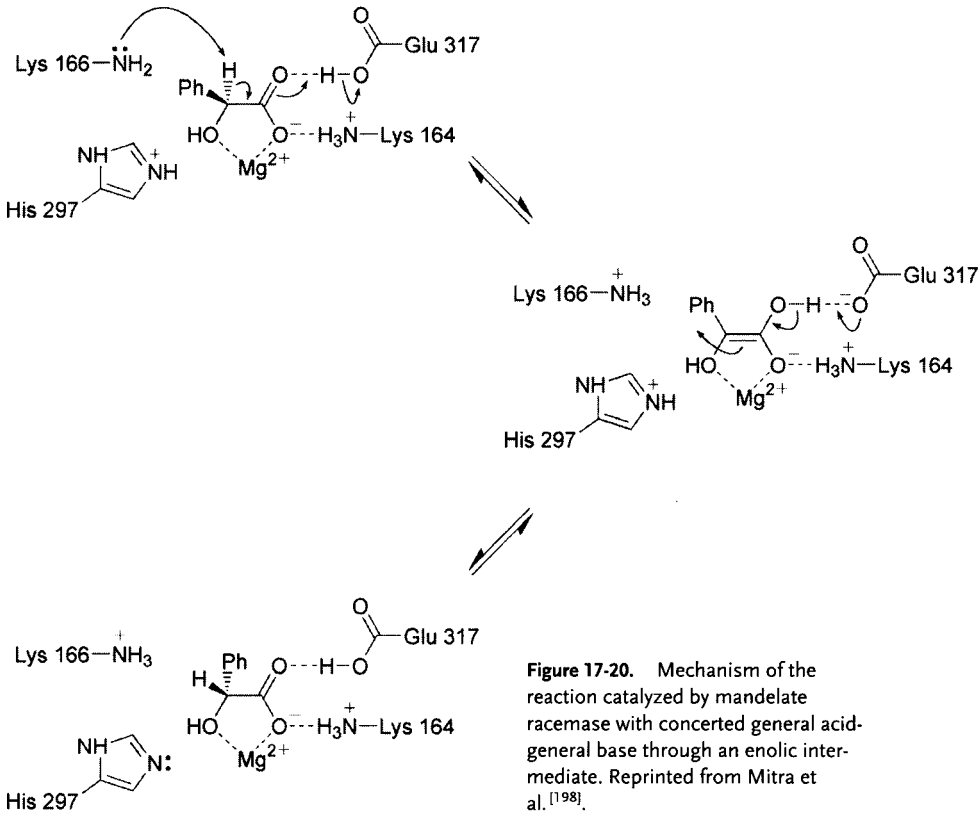


Figure 17-20. Mechanism of the reaction catalyzed by mandelate racemase with concerted general acid-general base through an enolic intermediate. Reprinted from Mitra et al.[198].

17.3.1
D-Xylose (Glucose) Isomerase (E.C. 5.3.1.5)

D-Xylose isomerase catalyzes the interconversion between D-xylose and D-xylulose (Fig. 17-21). Since this enzyme acts on D-glucose to produce D-fructose, it is often referred to as glucose isomerase (Fig. 17-21). The isomerization of glucose to fructose by this enzyme is a very important process for the industrial production of high fructose corn syrup. This enzyme is also applicable to the synthesis of many aldoses and ketoses because of its wide substrate specificity. The enzyme gene has been cloned from various microorganisms, and the enzyme has been overexpressed, purified, and characterized. Their three dimensional structures have also been determined[203–206].

17.3.1.1
Properties

Xylose isomerases have been purified from various microorganisms, such as *Lactobacillus brevis*, *Streptomyces* sp., *Bacillus stearothermophilus*, and *Actinoplanes*

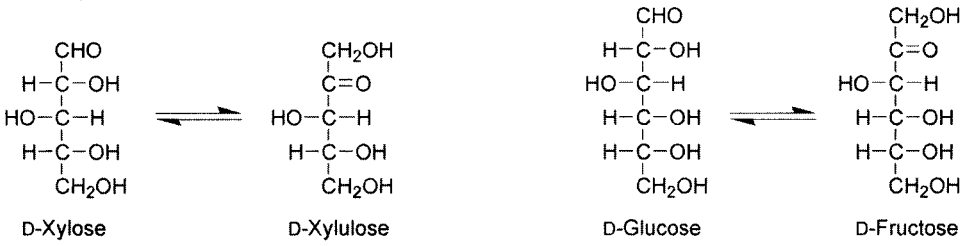


Figure 17-21. Reactions catalyzed by D-xylose isomerase.

missouriensis[207–210]. They consist of four identical subunits whose molecular mass are in the range 42 000–51 000. The optimum pH usually ranges from 7.0 to 9.0. The cDNA for barley (*Hordeum vulgare*) enzyme gene has been cloned, and the recombinant enzyme characterized[211]. It is unique because it is a dimer composed of a subunit with a molecular mass of 53 620, which is much larger than those of microbial enzymes. Thermostable xylose isomerases were purified and characterized from many thermophilic bacteria[204, 205, 212–222]. The enzyme isolated from *Thermotoga neapolitana* is extremely thermostable, with the optimal activity being above 95 °C[216]. The catalytic efficiency (k_{cat}/K_M) of the enzyme is essentially constant between 60 and 90 °C, and decreases between 90 and 98 °C primarily because of a large increase in K_M. Xylose isomerase requires divalent metal cations, usually Mg^{2+}, Mn^{2+}, or Co^{2+} for the maximum activity and thermal stability. The enzyme has a wide substrate specificity[223]: glucose and fructose derivatives modified at the 3-, 5- or 6-position are isomerized by the enzyme as will be described later.

17.3.1.2
Reaction Mechanism

The reaction mechanism of xylose isomerase was proposed based on X-ray crystallography[224] and molecular mechanical and molecular orbital studies[225].

The α-pyranose form of the substrate binds to the active site of the enzyme, and the reaction is initiated by ring-opening involving hydrogen transfer from the first hydroxyl group to O5 (Fig. 17-22). After extension of the substrate, a water molecule abstracts the proton from the hydroxyl group at O2 of xylose and transfers it to Asp 257 in the second step. The following hydride shift causes isomerization. The O1 atom of the ketose is negatively charged and most probably abstracts a proton from Asp 257. The stable cyclic conformation is then formed.

This hydride shift reaction mechanism is quite different from the base-catalyzed enolization mechanism proposed for phospho sugar isomerases such as triosephosphate isomerase which generally do not require a metal ion for activity[226].

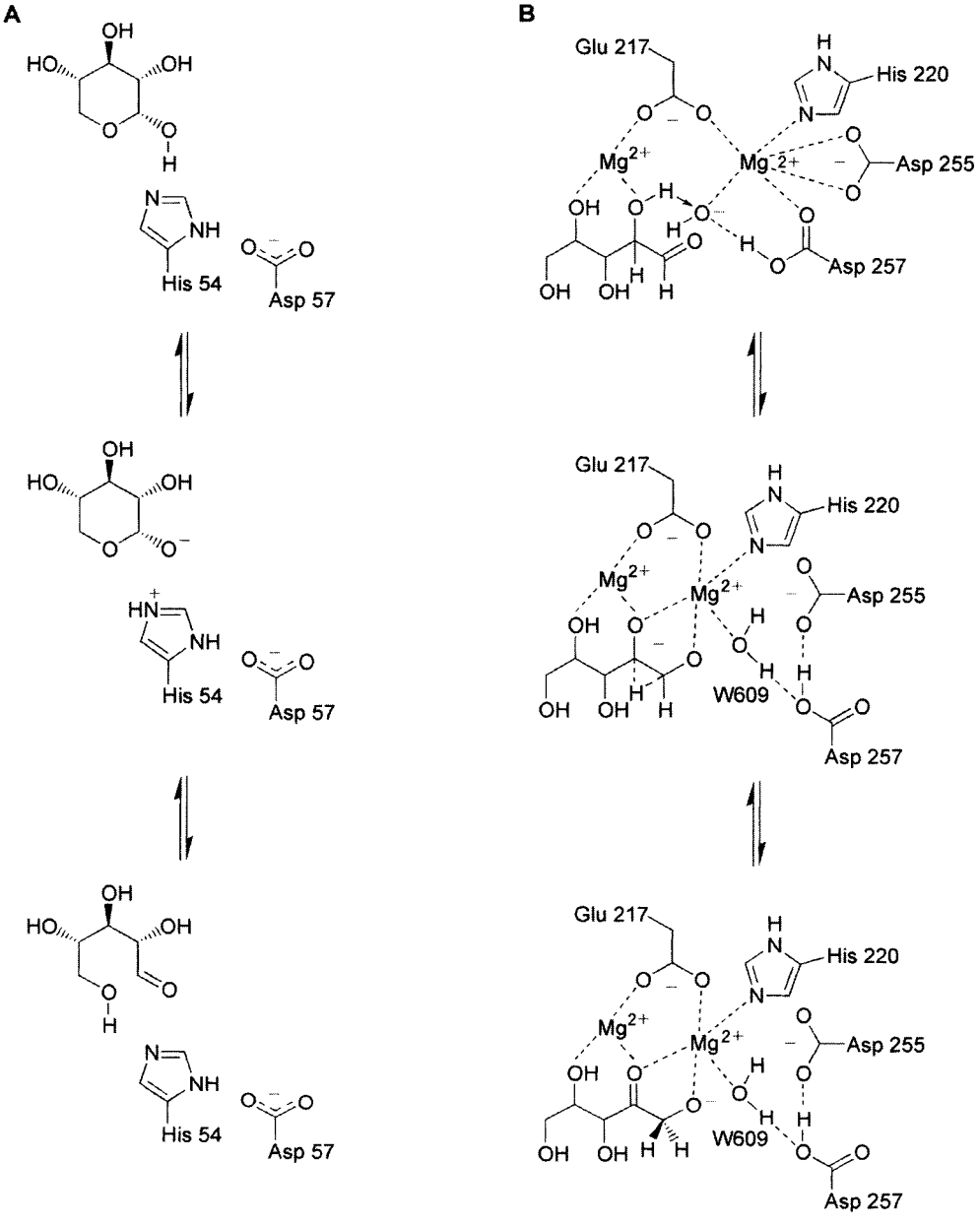


Figure 17-22. Reaction mechanism for xylose-xylulose conversion by D-xylose isomerase through ring opening (A) and hydride shift (B). Reprinted from Fuxreiter et al.[225].

17.3.1.3
Production of Fructose

Xylose isomerase derived from various microorganisms, such as *Actinoplanes missouriensis*, *Streptomyces griseofuscus*, *Flavobacterium arborescens*, *Streptomyces phaechromogenes*, *Bacillus coagulans*, *Streptomyces murinus*, *Streptomyces rubiginosus*, and *Streptomyces olivochromogenes*, is utilized in the annual conversion of 3 million tons of glucose into fructose for use as high fructose corn syrup. The enzyme is immobilized by glutaraldehyde cross-linking or adsorption on an insoluble resin for the fixed bed isomerization process [227].

The isomerization is reversible, and the final fructose content depends on the reaction temperature. The reaction is usually carried out in the region of 60–65 °C. However, a higher temperature gives a higher fructose content. It is reported that the degree of conversion is raised from 42 %, which is the normal fructose content of the syrup, to 55 % by isomerization with xylose isomerase at about 95 °C [227]. Therefore, the thermostability of the enzyme is an important issue. Recently, several thermostable xylose isomerases were found and characterized [204, 205, 212–222]. It is also reported that the thermostability of the enzyme is enhanced by site-directed mutagenesis [228].

α-Amylases and xylose isomerases with low optimum pH values are expected to be useful for fructose production from cornstarch because raw cornstarch solutions have an acidic pH of around 4.5 and the glucoamylase reaction, the second step in the process, prefers an acidic pH. Fructose can be produced from cornstarch without pH adjustment throughout the process at acidic pH values by means of such acidophilic α-amylases and xylose isomerases. Takasaki et al. [229] found an acidophilic α-amylase in a *Bacillus licheniformis* strain isolated from soil, and showed that the enzyme is suitable for digestion of cornstarch at an acidic pH of 4.5–5.0. Acidophilic xylose isomerases have been demonstrated in *Thermoanaerobacterium* sp. JW/SL-YS [217] and *Streptomyces* sp. SK [221], and purified and characterized. Both of these have optimum pH values around 6.5, but are highly active at acidic pHs such as 5.0. Since they are highly thermostable, they are expected to be useful for fructose production.

17.3.1.4
Production of Unusual Sugar Derivatives

Xylose isomerase has a wide substrate specificity, and 3-, 5-, or 6- substituted glucose and fructose are isomerized by this enzyme. Since this enzyme requires the 4-OH group for hexoses to be substrates, phosphoglucose isomerase instead of xylose isomerase is used for the synthesis of 4-substituted fructose as described below.

17.3.1.4.1 Preparation of Glucose Derivatives Modified at Position 3 or 6

Bock and coworkers [230] showed that D-glucose derivatives bearing modifications at the C3 or C6 position are converted by xylose isomerase from *Streptomyces* sp.

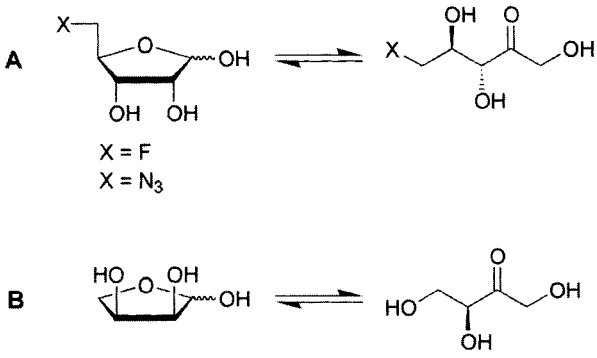


Figure 17-23. Conversion by xylose isomerase of (2*R*,3*R*)-configured aldotetrose modified at C5 into open-chain 2-ketoses (A), and L-erythrose into L-erythrulose (B). Reprinted from Ebner and Stütz[232].

However, epimers of D-glucose are inert as substrates of the enzyme: D-mannose, D-allose, and D-galactose. Various 5-modified D-glucofuranoses are quantitatively converted into the corresponding D-fructopyranoses with the enzyme[231]. Ebner and Stütz[232] showed that various (2*R*,3*R*)-configured aldofuranoses such as D-erythrose and C5-modified D-ribose derivatives serve as substrates of the enzyme: D-erythrose is quantitatively converted into D-glycero-tetrulose, with D-ribofuranoses being the corresponding open-chain 2-ketoses (Fig. 17-23). L-Erythrose, the enantiomer of D-erythrose, is also isomerized quantitatively by the enzyme to L-erythrulose (L-*glycero*-tetrulose) (Fig. 17-23). Fructose bisphosphate aldolase catalyzes a stereospecific aldol condensation between dihydroxyacetone phosphate and a number of aldehydes to form hexoketose 1-phosphates, the phosphate groups of which are removed by hydrolysis. The resultant hexoketoses are converted stereospecifically into hexoaldose derivatives by xylose isomerase. Thus, unusual hexoaldose derivatives such as 3-deoxy-D-glucose, 6-deoxy-D-glucose, 6-*O*-methyl-D-glucose and 6-deoxy-6-fluoro-D-glucose were prepared by this method[223, 233].

17.3.1.4.2 Preparation of Fructose and Sorbose Derivatives Modified at Position 5

Xylose isomerase converts a wide range of D-glucose as well as L-idose derivatives modified at position 5 into the corresponding ketose. 5-Deoxy-5-fluoro-D-xylulose and a variety of 5,6-dimodified open-chain analogs of D-fructose, namely the 5,6-diazido-5,6-dideoxy, 6-azido-5,6-dideoxy, 6-azido-5,6-dideoxy-5-fluoro, 5,6-dideoxy-5-fluoro, 5,6-dideoxy-6-fluoro and 5,6-dideoxy-5,6-difluoro derivatives were prepared with glucose isomerase (Fig. 17-24)[234, 235].

17.3.1.4.3 Preparation of Sucrose Derivatives with Modified Fructose Moieties

Xylose isomerase is also used for the synthesis of modified sucroses, which is important in the study of the topographical aspects of the binding of sucrose to a sucrose carrier protein[236]. 6-Deoxy- and 6-deoxy-6-fluoroglucose chemically synthesized are isomerized to the corresponding 6-substituted fructose by xylose isomerase. The resultant substrates are subsequently condensed with UDP-glucose by sucrose synthase. Although the equilibrium of the first step lies towards the glucose

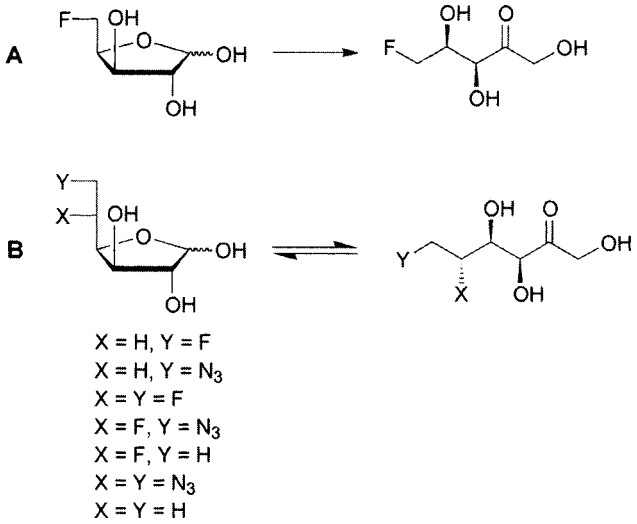


Figure 17-24. Production of 5-deoxy-5-fluoro-D-xylulose and 5,6-dimodified open-chain analogs of D-fructose with xylose isomerase. Reprinted from Hadwiger et al.[235].

derivatives, this problem is overcome by coupling the isomerization reaction with the sucrose formation, which is irreversible. The second reaction completely drives the isomerization reaction almost to completion. Incubation of 6-deoxy- or 6-deoxy-6-fluoroglucose and UDP-glucose with both the xylose isomerase and sucrose synthase afforded 6'-deoxy- and 6'-deoxy-6'-fluorosucrose in 73 and 53% isolated yield, respectively.

17.3.2
Phosphoglucose Isomerase (E.C. 5.3.1.9)

Phosphoglucose isomerase catalyzes the interconversion of glucose 6-phosphate and fructose 6-phosphate. This enzyme is involved in the gluconeogenesis, glycolytic pathway, and pentose phosphate cycle. Since thermostable enzymes are generally useful for industrial application, thermostable phosphoglucose isomerase was purified from *Bacillus stearothermophilus*[237] and *Bacillus caldotenax*[238]. *B. stearothermophilus* produces two isozymes of phosphoglucose isomerase, and they were overexpressed in *E. coli*, purified to homogeneity, crystallized[239], and the X-ray structure of the enzyme was determined[240, 241]. The structure of the rabbit muscle enzyme complexed with a competitive inhibitor D-gluconate 6-phosphate was also determined by X-ray crystallography[242, 243]. The enzyme is a dimer with two α/β-sandwich domains in each subunit. Lys 518 and His 388 are located at the active center and are probably involved in the catalytic mechanism. Since gluconate 6-phosphate occurs predominantly in its cyclic form, phosphoglucose isomerase probably catalyze the opening of the hexose ring to give initially its straight chain form with Lys 518 and His 388. Then the enzyme undergoes isomerization of the

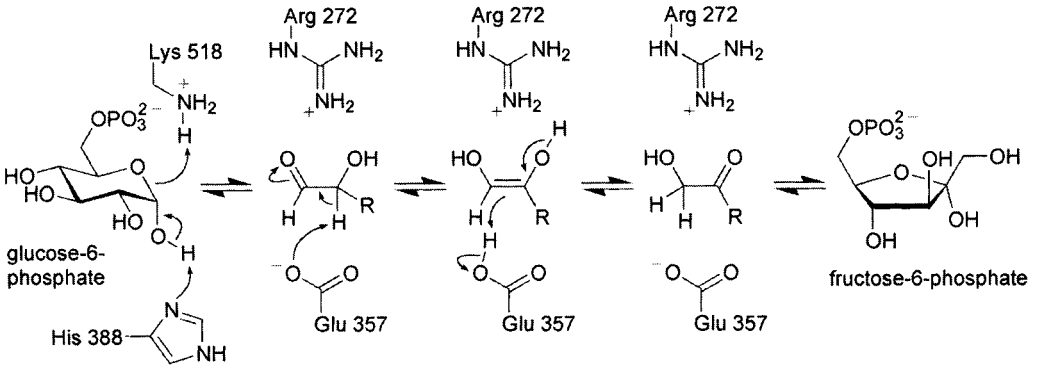


Figure 17-25. Mechanism of phosphoglucose isomerase reaction. His 388 and Glu 216 catalyze the ring opening. The side-chain of Glu357 abstracts a proton from the C2 position of the open chain form of the substrate, and the *cis*-enediol is formed. Then, a proton is transferred from the protonated Glu 357 to the C1 position of the intermediate. Reprinted from Jeffery et al.[242].

substrate through formation of a *cis*-enediol intermediate with the double bond between C1 and C2 (Fig. 17-25). Glu 357 transfers the proton from the C2 of glucose 6-phosphate to its C1 position. The side chain of Arg 272 stabilizes the negative charge of the intermediate (Fig. 17-25).

Xylose isomerase requires the 4-OH group for glucose derivatives to be substrates[230]. On the other hand, phosphoglucose isomerase can act on 4-substituted phosphoglucose. Therefore the latter enzyme is applicable to the preparation of glucose or fructose derivatives modified at position 4. For example, 4-deoxy-4-fluorofructose was prepared from 4-deoxy-4-fluoroglucose with phosphoglucose isomerase because xylose isomerase cannot isomerize 4-deoxy-4-fluoroglucose[236]. 4-Deoxy-4-fluorofructose was then converted into 4'-deoxy-4'-fluorosucrose, which is useful for the analysis of the interaction between sucrose and a sucrose carrier protein, with fructose-6-phosphate kinase[236].

Fructose 1,6-bisphosphate has attracted attention due to its important applications in the field of medicine, and is produced from glucose in three step by enzymatic reactions catalyzed by glucokinase, phosphoglucose isomerase, and phosphofructokinase. ATP is regenerated by acetate kinase (Fig. 17-26). Ishikawa and coworkers established an efficient method for production of fructose 1,6-bisphosphate in a

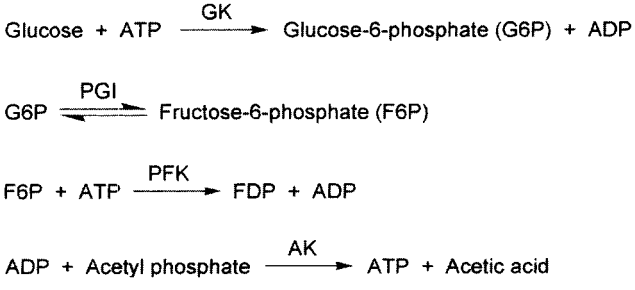


Figure 17-26. Synthesis of fructose 1,6-bisphosphate from glucose by combination of glucokinase (GK), phosphoglucose isomerase (PGI), phosphofructokinase (PFK), and acetate kinase (AK) reactions.

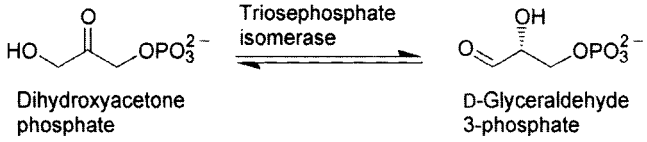


Figure 17-27. Reaction catalyzed by triosephosphate isomerase.

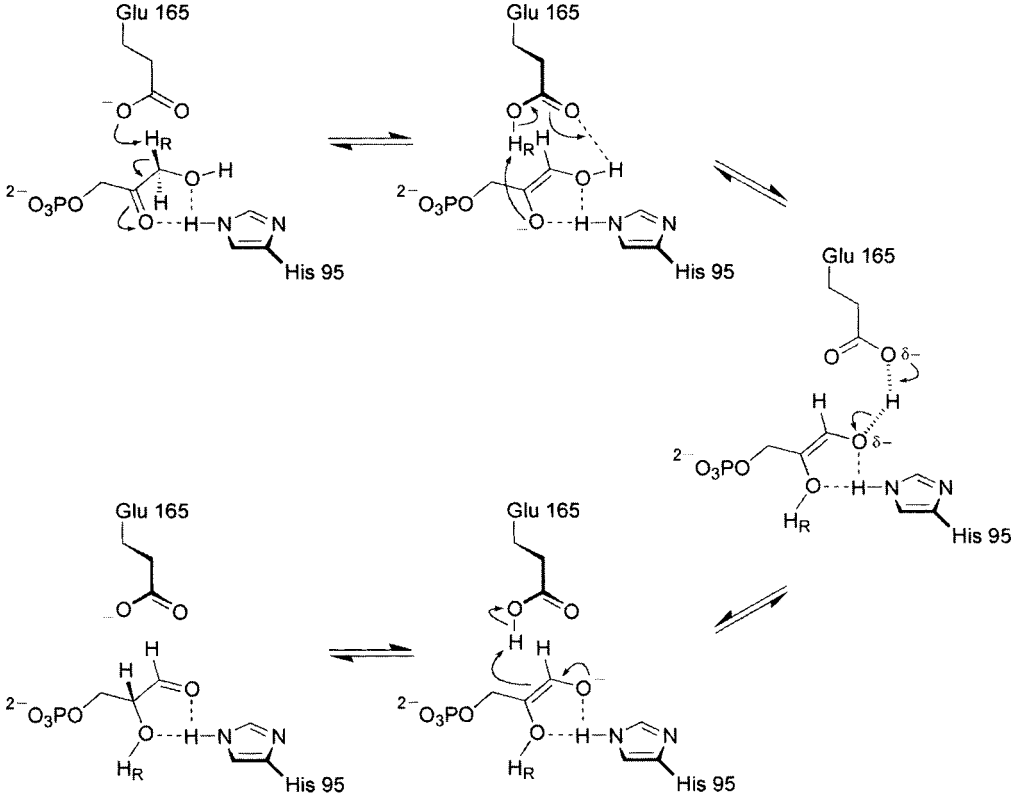


Figure 17-28. Triosephosphate isomerase reaction through a *cis*-enediol intermediate. The *pro-R* proton is removed from C1 of dihydroxyacetone phosphate by the side chain of Glu 165, and the carbonyl group of the substrate is polarized by the side chain of His 95. Reprinted from Harris et al.[249].

batch reactor system using the purified enzymes[244] and the crude extract of *Bacillus stearothermophilus* cells[245]. The yield of fructose 1,6-bisphosphate depended on the activity of glucokinase in the reactor[246].

17.3.3
Triosephosphate Isomerase (E.C. 5.3.1.1)

Triosephosphate isomerase is involved in the glycolytic pathway, and catalyzes the interconversion of dihydroxyacetone phosphate and D-glyceraldehyde phosphate (Fig. 17-27). The refined three-dimensional structures of chicken, yeast, and trypano-

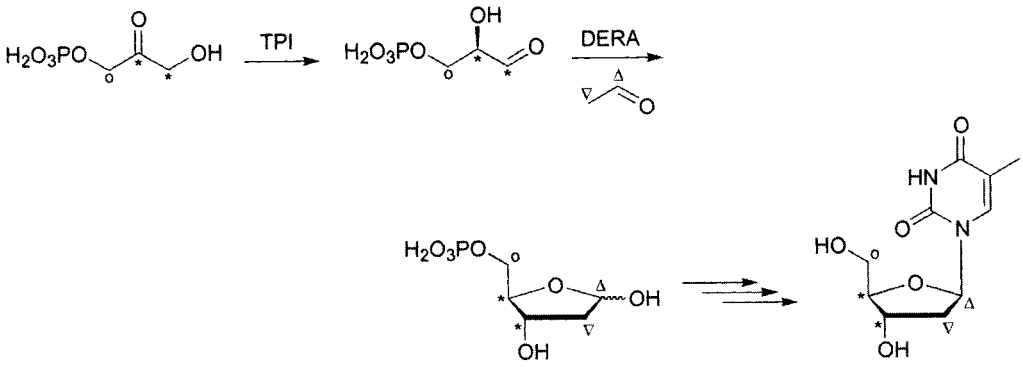


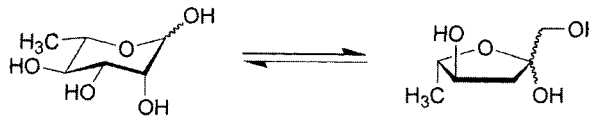
Figure 17-29. Synthesis of [3',4'-$^{13}C_2$]-thymidine from [2',3'-$^{13}C_2$]-dihydroxyacetone phosphate with triosephosphate isomerase (TPI) and D-2-deoxyribose-5-phosphate (DHAP). Asterisks indicate the positions selectively labeled with ^{13}C. Other positions that can be isotopically substituted are marked with °, △, and ▽. Reprinted from Ouwerkerk et al.[251].

somal enzymes have been elucidated[247]. The reaction is thought to proceed through a *cis*-enediol intermediate with Glu 165 and His 95 as acid and base catalysts (Fig. 17-28)[248, 249]. The side chain of Glu 165 removes the *pro-R* proton from the C1 of dihydroxyacetone phosphate, and that of neutral His 95 polarizes the carbonyl group of the substrate. Fructose 1,6-bisphosphate, a precursor molecule for sugar synthesis, can be prepared from dihydroxyacetone phosphate with this enzyme and aldolase[250]. Triosephosphate isomerase has been used for various other purposes. For example, [3',4'-$^{13}C_2$]-thymidine has been prepared from [$^{13}C_2$]-acetic acid through [2',3'-$^{13}C_2$]-dihydroxyacetone phosphate and D-[3',4'-$^{13}C_2$]-2-deoxyribose-5-phosphate with triosephosphate isomerase and D-2-deoxyribose-5-phosphate aldolase (E.C. 4.2.1.2) (Fig. 17-29)[251].

17.3.4
L-Rhamnose Isomerase (E.C. 5.3.1.14)

L-Rhamnose is an important component of bacterial cell walls, and is metabolized in *E. coli* through a pathway similar to that of glucose 6-phosphate in glycolysis. Rhamnose isomerase catalyzes the first reaction in the pathway to produce L-rhamnulose from L-rhamnose (Fig. 17-30). The enzyme gene was cloned from *E. coli* and overexpressed[252], and the enzyme was purified and characterized[252].

Rhamnose isomerase is composed of four identical subunits with a molecular mass of about 47 kDa. It has the maximum activity around 7.6, and requires Mn^{2+} to provide the highest activity. The enzyme shows no significant sequence similarity to any other ketol isomerases including xylose isomerase. However, rhamnose isomerase was found, by X-ray crystallography, to be most similar to xylose isomerase[252]. The monomer of rhamnose isomerase is composed of $(\beta/\alpha)_8$-barrels, and the structure and arrangement of the barrel are very similar to those of xylose isomerase. However, each of them has an additional α-helical domain, which is involved in subunit assembly and differs from each other only in its structure. The



L-Rhamnose
(6-Deoxy-L-mannose)

L-Rhamnulose
(6-Deoxy-L-fructose)

Figure 17-30. Reaction catalyzed by rhamnose isomerase. Since both substrate and product occur in cyclic forms, L-rhamnose isomerase catalyzes ring opening before isomerization. Reprinted from Korndorfer et al.[252].

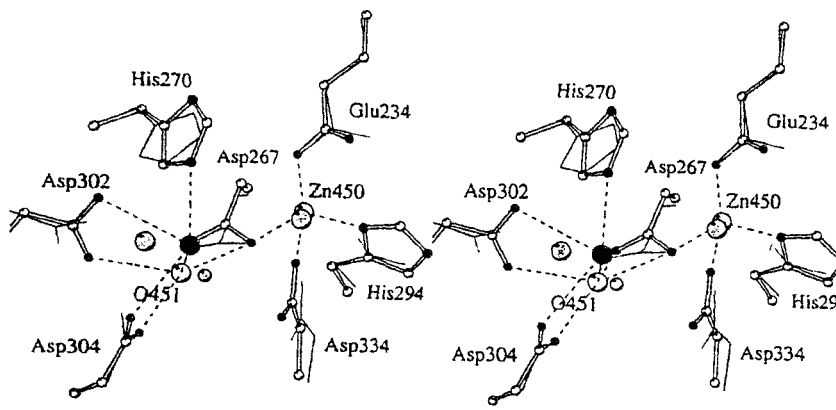


Figure 17-31. Superposition of the metal binding sites of rhamnose isomerase (residues named and drawn with thick bonds) and zylose isomerase (thin bonds). Reprinted from Korndorfer et al.[252].

residues surrounding the catalytic Mn^{2+} site (Asp 302, Asp 304 and His 270) are conserved in the two structures (Fig. 17-31). Therefore, the reaction catalyzed by rhamnose isomerase is thought to proceed through a metal-mediated hydride-shift mechanism in the same manner as xylose isomerase[252].

Bhuiyan et al.[253] immobilized L-rhamnose isomerase from *Pseudomonas* sp. LL172 on chitopearl beads, and used it to produce L-mannose from L-fructose. The immobilized enzyme was found to be stable: it retained about 90% of the initial activity after five repeated batch reactions. The concentration of L-mannose relative to L-fructose was about 3:7 at equilibrium. D-Allose was also produced from D-psicose with the immobilized L-rhamnose isomerase. Since D-psicose is readily produced from D-fructose with D-tagatose 3-epimerase, D-allose can be produced from D-fructose by combination of the two enzymes immobilized on chitopearl beads. Bhuiyan et al.[254] found that the reaction progresses steadily until 40% of the D-psicose is converted into D-allose. The immobilized D-tagatose 3-epimerase was also stable even after repeated uses, and D-allose was produced efficiently in the system.

17.3.5
L-Fucose Isomerase (E.C. 5.3.1.3)

Fucosylated oligosaccharides are important components of glycoproteins and glycolipids which are useful for cancer diagnosis and immunotyping. Therefore, efficient production methods for L-fucose and its analogs would be useful.

L-Fucose isomerase acts on D-arabinose, which was known as D-arabinose isomerase in earlier literatures. L-Fucose is metabolized through a pathway similar to that of D-glucose in glycolysis, and L-fucose isomerase corresponds to glucose 6-phosphate isomerase. However, none of the aldose-ketose isomerases including glucose 6-phosphate isomerase shows sequence similarity to L-fucose isomerase. L-Fucose isomerase shares the common characteristics with other aldose-ketose isomerases acting on unphosphorylated substrates: the requirement of metal ions such as Mn^{2+} for L-fucose isomerase. Aldose-ketose isomerases acting on phosphorylated substrates generally require no metal ions with the exception of phosphomannose isomerase (E.C. 5.3.1.8) which requires Zn^{2+} for its activity.

Seemann and Schulz[255] determined the three-dimensional structure of L-fucose isomerase from *E. coli*, a hexamer from a subunit with a molecular mass of 64 976 Da. The enzyme shows no structural similarity to any other aldose-ketose isomerases analyzed thus far. However, Seemann and Schulz, on the basis of the tertiary structure, suggested that the L-fucose isomerase reaction proceeds through an enediol intermediate[255].

Fessner et al.[256] developed an efficient method for the synthesis of L-fucose analogs modified at the nonpolar terminus by means of L-fucose isomerase and L-fuculose 1-phosphate aldolase from *E. coli*. Various L-fucose analogs bearing linear or branched aliphatic side chains were prepared in about 30% overall yield with hydroxyaldehyde precursors and dihydroxyacetone phosphate as the starting materials (Fig. 17-32).

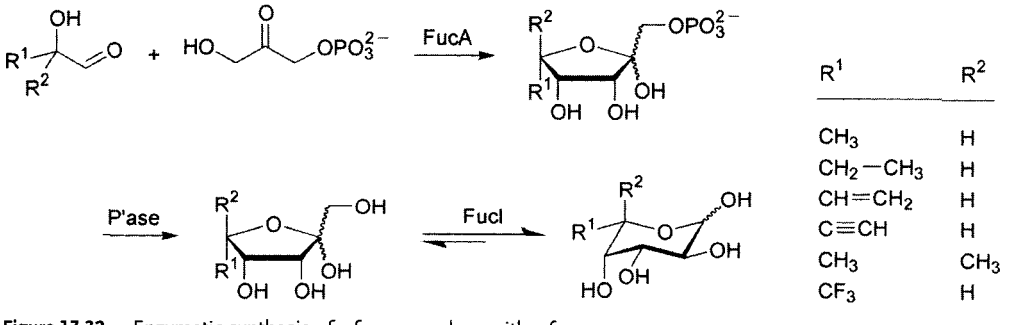


Figure 17-32. Enzymatic synthesis of L-fucose analogs with L-fucose 1-phosphate aldolase (FucA), phosphatase (P'ase), and L-fucose isomerase (FucI). Reprinted from Fessner et al.[256].

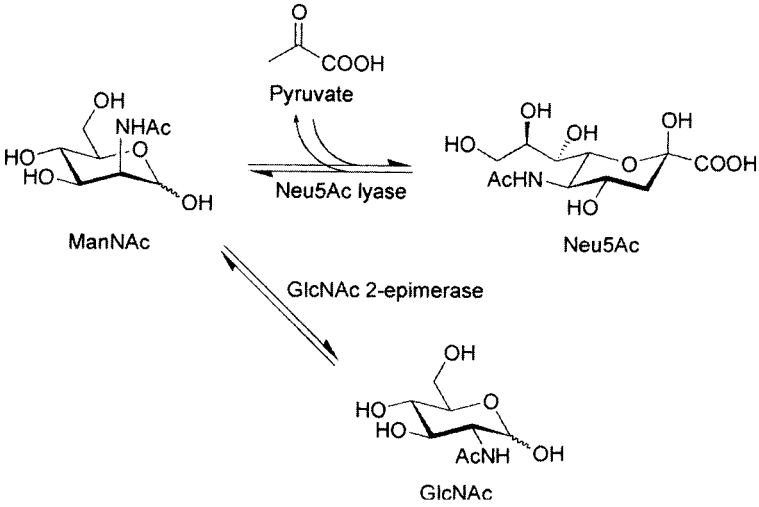


Figure 17-33. Synthesis of *N*-acetylneuraminate (Neu5Ac) from *N*-acetyl-D-glucosamine (GlcNAc) and pyruvate through *N*-acetyl-D-mannosamine (ManNAc) with *N*-acetylneuraminate and *N*-acetyl-D-glucosamine 2-epimerase. Reprinted from Maru et al.[259].

17.3.6
N-Acetyl-D-glucosamine 2-Epimerase

N-Acetylneuraminate is a sialic acid with various biological functions that is widely distributed in animals. It has been prepared only from natural resources such as colominic acid, edible birds nests, milk or eggs. Alternatively, it has been prepared enzymatically from *N*-acetyl-D-mannosamine and pyruvate with *N*-acetylneuraminate lyase as the catalyst[257, 258]. However, *N*-acetyl-D-mannosamine is expensive, and the method is not suitable for large-scale production of *N*-acetylneuraminate. Maru et al.[259] developed an elegant method for the enzymatic production of *N*-acetylneuraminate from the inexpensive *N*-acetyl-D-glucosamine and pyruvate by means of *N*-acetylneuraminate lyase and *N*-acyl-D-glucosamine 2-epimerase, whose genes were cloned from *E. coli*[260] and pig kidney[261], respectively (Fig. 17-33). Simultaneous use of these enzymes and feeding of appropriate amounts of pyruvate to the reaction mixture enabled production of *N*-acetylneuraminate from *N*-acetyl-D-glucosamine with a 77% conversion rate, and 29 kg of *N*-acetylneuraminate were obtained from 27 kg of *N*-acetyl-D-glucosamine.

17.3.7
Maleate *cis-trans* Isomerase (E.C. 5.2.1.1)

Maleate *cis-trans* isomerase catalyzes the conversion of maleate into fumarate. This enzyme is applicable to the production of L-aspartate by coupling with the aspartase reaction as shown in Fig. 17-34[262, 263]. First, maleate is isomerized to fumarate by

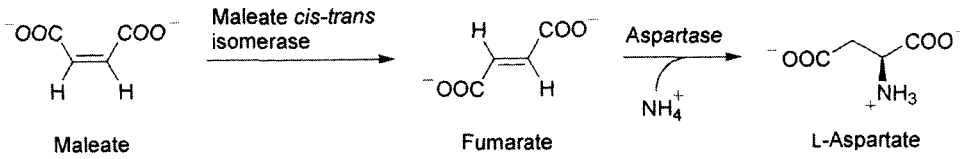


Figure 17-34. Synthesis of L-aspartate using maleate cis-trans isomerase and aspartase.

cis-trans isomerase, and then the fumarate formed is aminated to L-aspartate by aspartase. In this procedure, the resting cells of *Alcaligenes faecalis* containing both enzymes can be used as a catalyst. Thermostable maleate *cis-trans* isomerase was purified from *Bacillus stearothermophilus* MI-102 and characterized, and the enzyme gene was cloned and sequenced[264]. Two cysteine residues, Cys 80 and Cys 198, among the three conserved cysteines were found by site-directed mutagenesis studies to be catalytically important, although their catalytic roles are not yet known.

17.3.8
Unsaturated Fatty Acid *cis-trans* Isomerase

trans-Unsaturated fatty acids occur in membrane phospholipids of some bacterial genera such as *Pseudomonas* and *Vibrio*[265]. They are produced by *cis-trans* isomerase from *cis*-unsaturated fatty acids in response to environmental stresses such as elevated temperatures, increased salt concentrations, and the presence of organic solvents such as toluene[266–269]. The structural gene for the *cis-trans* isomerase was cloned from *Pseudomonas putida* P8[270]. The *E. coli* recombinant cells carrying the gene were shown to produce *trans*-unsaturated fatty acids in response to the organic solvent, although *E. coli* has no inherent ability to produce these fatty acids[270].

Okuyama et al.[271] purified the *cis-trans* isomerase from *Pseudomonas* sp. E-3 and characterized the enzyme catalyzing *cis-trans* isomerization toward 9-hexadecenoate. It catalyzes the *cis*-to-*trans* conversion of a double bond of *cis*-mono-unsaturated fatty acids with carbon chain lengths of 14, 15, 16, and 17 at positions 9, 10, or 11, but not at 6 or 7: the enzyme shows a strict specificity for both the position of the double bond and the chain length of the fatty acid. A similar enzyme was also discovered by Witholt and coworkers, which was purified from the periplasmic fraction of *Pseudomonas oleovorans*[272]. Not only 9-*cis*-hexadecenoate but also 11-*cis*-octadecenoate were found to serve as substrates of the enzyme. Moreover, the enzyme acted only on free unsaturated fatty acids and not on esterified fatty acids in contrast to the enzyme from *Pseudomonas* sp. E-3. Therefore, the *Pseudomonas oleovorans* enzyme differs from the enzyme of *Pseudomonas* sp. E-3 in substrate specificity, although both are monomeric enzymes with a molecular mass of about 80 kDa. The *cis-trans* isomerases are expected to be useful for biotransformation of unsaturated fatty acids.

17.4 Conclusion

Total conversion of racemic starting materials into a particular stereoisomer of a desired compound is very useful in the chemical industry. Half or more of the starting materials can be saved and steps for the laborious separation of the products from the starting material remaining reduced. Thus, racemases and epimerases are very useful in the chemical industry, when their reactions are coupled with some stereospecific reactions. Isomerases are also powerful catalysts for the production of particular enantiomers or diastereomers of interest from cheaply-available starting materials especially in the field of carbohydrate chemistry. Various new racemases and isomerases useful for industrial applications will no doubt be discovered from microorganisms at some point. However, established and well-known enzymes can be remodeled in order to expand their uses by various protein engineering technologies such as directed evolution. A good example for this is L-specific hydantoinase derived from D-specific hydantoinase[154]. The engineered enzymes can be incorporated into metabolic engineering studies in order to develop powerful microbial cells.

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18
Introduction and Removal of Protecting Groups

Dieter Kadereit, Reinhard Reents, Duraiswamy A. Jeyaraj and Herbert Waldmann

18.1
Introduction

The proper introduction and removal of protecting groups is one of the most important and widely carried out synthetic transformation in preparative organic chemistry. In particular, in the highly selective construction of complex, polyfunctional molecules, e. g. oligonucleotides, oligosaccharides, peptides and conjugates thereof, and in the synthesis of alkaloids, macrolides, polyether antibiotics, prostaglandins and other natural products, regularly the problem arises that a given functional group has to be protected or deprotected selectively under the mildest conditions and in the presence of functionalities of similar reactivity, as well as in the presence of structures that are sensitive to acids, bases, oxidation and reduction. Numerous classical chemical methods have been developed for the manipulation of protecting groups [1–3]. Nevertheless, severe problems still remain caused by the need to introduce or remove selectively specific blocking functions which can not, or only with great difficulties, be solved by using classical chemical tools only. However, the arsenal of the available protecting group techniques has been substantially enriched by the application of biocatalysts. In addition to their stereodiscriminating properties, enzymes offer the opportunity to carry out highly chemo- and regioselective transformations. They often operate at neutral, weakly acidic or weakly basic pH values and in many cases combine a high selectivity for the reactions they catalyze and the structures they recognize with a broad substrate tolerance. Therefore, the application of these biocatalysts to effect the introduction and/or removal of suitable protecting groups offers viable alternatives to classical chemical methods [4–11].

18.2
Protection of Amino Groups[4–12]

18.2.1
N-Terminal Protection of Peptides

The selective protection and liberation of the α-amino function, the carboxy group and the various side chain functionalities of polyfunctional amino acids constitute some of the most fundamental problems in peptide chemistry. Consequently, numerous efficient protective functions based on chemical techniques have been developed to a high level of practicability.[1–3, 13, 14] However, since the mid-1970s, a systematic search for blocking groups being removable with a biocatalyst has been carried out[4–12]. In addition to the mild deprotection conditions they promise, protecting groups of this type are expected to be particularly useful for the construction and manipulation of larger peptide units, i.e. for transformations which, for solubility reasons, in general have to be carried out in aqueous systems. Also applications in the reprocessing of peptides obtained by recombinant DNA technology are foreseen (for an interesting appropriate example see Chapter 12.5).

Initial attempts to introduce an enzyme-labile amino protecting group involved the use of chymotrypsin for the removal of *N*-benzoylphenylalanine (Bz-Phe) from the tripeptide Bz-Phe-Leu-Leu-OH[15]. The desired dipeptide H-Leu-Leu-OH was obtained in 80% yield under mild conditions (pH 7.3, room temperature). Chymotrypsin, however, is an endopeptidase with a rather broad substrate tolerance, catalyzing the hydrolysis of peptide bonds on the carboxy groups of hydrophobic and of aromatic amino acid residues. Since such amino acids appear widely in peptides, and since no method is available to protect them against attack by the enzyme during the attempted deprotection, the use of chymotrypsin is problematic. Its use is therefore limited to special cases[16] in which no danger of competitive cleavage at undesired sites has to be feared. A protease of much narrower specificity is trypsin which catalyzes the hydrolysis of peptide bonds at the carboxylic group of lysine and arginine. These amino acids carry polar, chemically reactive side chain functional groups which can be protected by various techniques[13, 14]. The high specificity of trypsin together with the possibility of hiding the critical amino acids which function as primary points of tryptic cleavage allowed for the development of a broadly applicable system for the protection of the α-amino group of peptides[12, 17–19]. In several studies the application of trypsin-labile protecting groups, along with suitable blocking functions for the side chains of arginine and lysine were described[17–23]. Thus, for instance Z-Arg-OH served as the enzymatically removable protecting group in a stepwise synthesis of deamino-oxytocin 1 (Fig. 18-1)[18, 19].

Starting with a pentapeptide the amino acid chain was elongated with Z-Arg-protected amino acid *p*-nitrophenyl esters. The *N*-terminal Z-Arg protecting group was successively removed in moderate to high yield and without attack on the other peptide bonds by treatment with trypsin. Unfortunately, the preparation of the protected arginine *p*-nitrophenyl esters is difficult, thus preventing this method from becoming generally useful for the stepwise assembly of larger peptides. The trypsin-

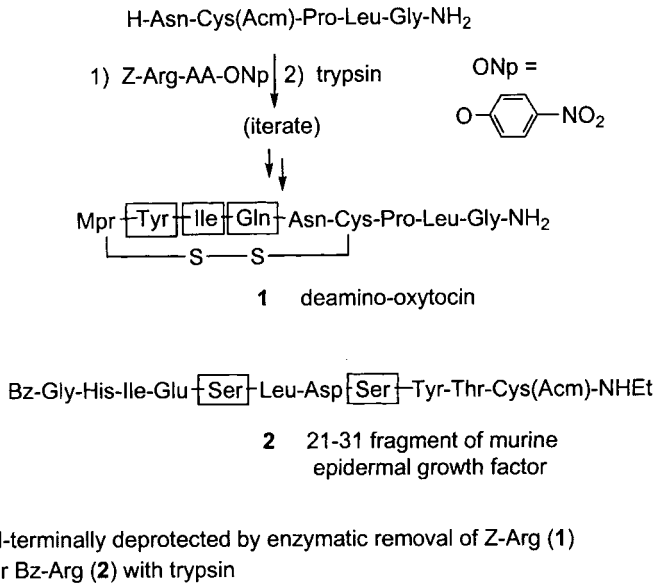


Figure 18-1. Construction of oligopeptides via removal of *N*-terminal arginine residues with trypsin.

labile blocking groups have, however, proven to be very useful for the construction of oligo- and polypeptides via condensation of preformed peptide fragments. An illustrative example consists of a chemoenzymatic construction of the 21–31 fragment **2** of murine epidermal growth factor (Fig. 18-1). In the course of this synthesis the deblocking by trypsin was applied twice[16]. The enzyme first liberated the *N*-terminus of a tetrapeptide and subsequently of a heptapeptide. In a synthesis[24] of human β-lipotropin an Ac-Arg-residue was introduced by a solid-phase technique at the *N*-terminus of the 29 *C*-terminal amino acids of the desired polypeptide. After cleavage from the resin and protection of the side chain functionalities, the arginine moiety was removed with trypsin, leaving the peptide chain intact. Finally, coupling of this 61–89 fragment to a partially protected 1–60 segment, and subsequent deprotection delivered β-lipotropin. Further examples are found in syntheses of oxypressin[12], Met-enkephalin[25] and Glu^4-oxytocin[12].

In addition to chymotrypsin and trypsin, the collagenase from *Clostridium histolyticum* has been proposed as a catalyst for the removal of *N*-terminally attached dummy amino acids from peptides[26]. The enzyme recognizes the tetrapeptides Pro-X-Gly-Pro and cleaves the X-Gly bond. The use of this biocatalyst permitted the construction of des-pyroglutamyl-[15-leucine]human little gastrin I by selective hydrolysis of the dipeptide Pz-Pro-Leu (Pz = 4-phenylazobenzyloxycarbonyl) from the *N*-terminus of the octadecapeptide Pz-Pro-Leu-Gly-Pro-Trp-Leu-$(Glu)_5$-Ala-Tyr-Gly-Trp-Leu-Asp-Phe-NH_2. Transformations of this type are analogous to the naturally occuring conversion of prohormones into hormones and may prove to be useful for the processing of peptide factors produced by recombinant DNA technology.

Despite the impressive syntheses that have been made possible using proteases, the use of these enzymes is always accompanied by the danger of a competitive (and sometimes unexpected and unforeseeable) cleavage of the peptide backbone at an undesired site. At a minimum, complex protecting group schemes may become necessary if the amino acid which serves as the recognition structure for the protease occurs several times in the peptide chain to be constructed. This disadvantage can be overcome if a biocatalyst devoid of peptidase activity is used for the liberation of the *N*-terminal amino group. This principle has been illustrated by the application of penicillin G acylase from *E. coli*[27–44] in industry for the large scale synthesis of semisynthetic penicillins and by using a phthalyl imidase from *Xanthobacter agilis*[45–47] (*vide infra*). Penicillin G acylase attacks phenylacetic acid (PhAc) amides and esters but does not hydrolyze peptide bonds. The acylase accepts a broad range of protected peptides as substrates and selectively liberates the *N*-terminal amino group under almost neutral conditions (pH 7–8, room temperature) leaving the amide bonds as well as the *C*-terminal methyl, allyl, benzyl and *tert*-butyl esters unaffected[28–35, 38]. The PhAc group is easily introduced into amino acids by chemical[48] or enzymatic[49] methods and is stable during the removal of the *C*-terminal protecting groups employed[29–32].

Recently, it has been shown that a phthalyl amidase isolated from *Xanthobacter agilis* is able to deprotect a variety of phthalimido substrates once the substrates are partially hydrolyzed to their monoacids (Fig. 18-2)[45–47]. The phthalyl group is commonly used for amine protection, because it completely blocks this functionality by double acylation[2, 3]. The enzymatic phthalyl removal proceeds via a two step process of weakly basic hydrolysis to yield the monoacid **4** and subsequent treatment with the phthalyl amidase (Fig. 18-2). Because the hydrolysis of the phthalimide **3** to the corresponding monoacid **4** can be catalyzed by imidases such as the rat liver imidase,[50] this procedure in particular represents a powerful alternative to the classical phthalyl deprotection which requires relatively drastic conditions and toxic reagents. However, the general applicability of the enzymatic phthalyl removal is yet to be investigated.

If the construction of PhAc- or phthalyl-peptides is carried out by chemical activation of the PhAc-amino acids, the application of the non-urethane blocking group results in ca. 6% racemization[29, 30]. However, this disadvantage can be overcome by forming the peptide bonds enzymatically, e.g. with trypsin[51], chymotrypsin[51] or carboxypeptidase Y[39, 51], or by using urethane-type protecting groups (*vide infra*). For such condensation reactions and the subsequent enzymatic removal of the PhAc group, a continuous process was developed which has the potential to be transferable to a larger scale[39].

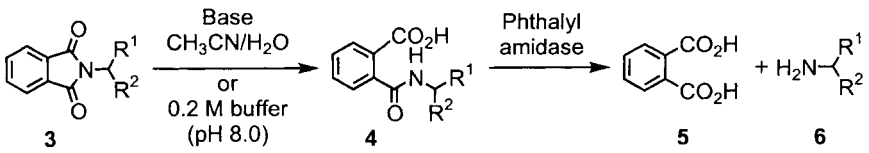
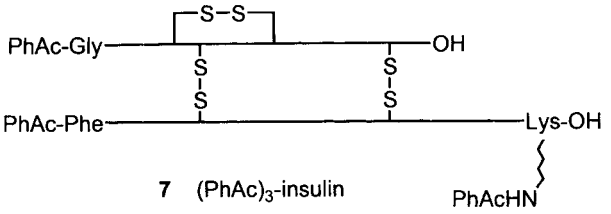
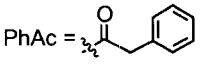
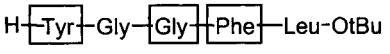


Figure 18-2. Enzymatic removal of the phthalyl group.



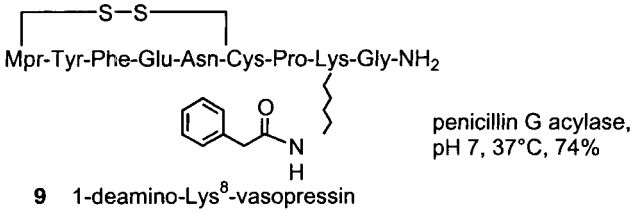
7 $(PhAc)_3$-insulin





8 leucine enkephalin

□ = N-terminally deprotected using penicillin G acylase



9 1-deamino-Lys^8-vasopressin

Figure 18-3. Application of the phenylacetamido (PhAc) group as an enzymatically removable amino protecting group.

The applicability of the penicillin acylase-catalyzed deprotection for the construction of larger peptides has been demonstrated by the complete deprotection of the porcine insulin derivative **7** carrying three PhAc groups[27], presumably at the *N*-terminal glycine of the A-chain, the *N*-terminal phenylalanine of the B-chain and the side chain of the lysine in position 29 of the B-chain (Fig. 18-3). The enzymatic hydrolysis proceeded to completeness and the peptide backbone was not attacked. A further interesting example is given by a recent biocatalyzed synthesis of leucine enkephalin *tert*-butyl ester **8**[38] in which all critical steps are performed by enzymes, two of them through the agency of penicillin G acylase: i) phenylacetates are introduced as *N*-terminal protecting groups of the amino acid esters by using penicillin G acylase, ii) the elongation of the peptide chain is carried out with papain or α-chymotrypsin, iii) the deprotection of the *N*-terminal amino group is achieved again by means of penicillin G acylase. These examples and also the application of this technique for aspartame syntheses[28, 40, 41], as well as the deprotection of glutathione derivatives[35] demonstrate that penicillin G acylase can be used advantageously for the *N*-terminal unmasking of peptides. In addition, the enzyme has

been used for the liberation of the side chain functionalities of lysine and cysteine, as well as in β-lactam, nucleoside and carbohydrate chemistry (*vide infra*).

18.2.2
Enzyme-labile Urethane Protecting Groups

The enzyme-labile *N*-protecting functions described so far are simple acyl groups which typify the danger of razemization during chemical peptide syntheses. This problem can, in general, be overcome by the use of urethane blocking functions. However, so far only few examples of a biocatalytic removal of classical urethane protecting groups such as the Z- and Boc-group are known[52]. Apparently, the enzymatic attack on the urethane carbonyl group, which would initiate the cleavage process, is too inefficient to be useful for synthetic purposes. To overcome this problem, two different strategies were developed. Both concepts have in common the fact, that the enzyme-labile bond is no longer part of the urethane. However, the first approach includes the introduction of a spacer (the AcOZ- and PhAcOZ groups), while the second strategy relies on the cleavage of a glycosidic C - O-bond of a glycoside urethane by the respective biocatalyst, e.g. a glucosidase (the BGloc group).

Through the introduction of a spacer between the group which is recognized by the enzyme and the urethane, the substrate is kept at a distance from the enzyme during the reaction (Fig. 18-4). Therefore, any steric effects caused by the bulk of certain amino acids are expected to be minimal and, as the amino acid sequence does not influence the reactivity, this concept should be generally applicable to the synthesis of peptides and peptide conjugates. An additional advantage of the introduction of the spacer is the option to choose the group that is recognized by the enzyme and thus the enzyme itself.

This concept was first realized by using p-hydroxybenzyl alcohol as a spacer in the *p*-(acetoxy)-benzyloxycarbonyl (AcOZ) group which encorporates an acetic acid ester as the enzyme-labile bond (Fig. 18-4). Accordingly, the AcOZ group can be removed under conditions typical for acetyl ester hydrolysis, for instance by treatment with lipases or esterases[53–55]. As lipases display a broad specificity, other esters present in the substrate molecule might be hydrolyzed during the AcOZ removal. Thus, the *p*-(phenylacetyl)benzyloxycarbonyl (PhAcOZ) group was developed, which takes advantage of the high selectivity of penicillin G acylase for the phenylacetyl group (Fig. 18-4). The versatiliy of this enzyme-labile urethane protecting group was demonstrated by the synthesis of phosphorylated[56–60], glycosylated[56–60] and lipidated[61] peptides.

A second approach takes advantage of a characteristic property of glycosidases. It is well known that glycosidases hydrolyze their substrates by cleaving the glycosidic bond via nucleophilic attack at the anomeric carbon atom. Therefore, a carbohydrate-derived urethane protecting group would provide the desired enzyme-lability. In additional, such sugar derivatives have increased solubility in aqueous solutions, a necessary requirement for all biotransformations. This concept was successfully realized by using glucose and galactose as the carbohydrate component

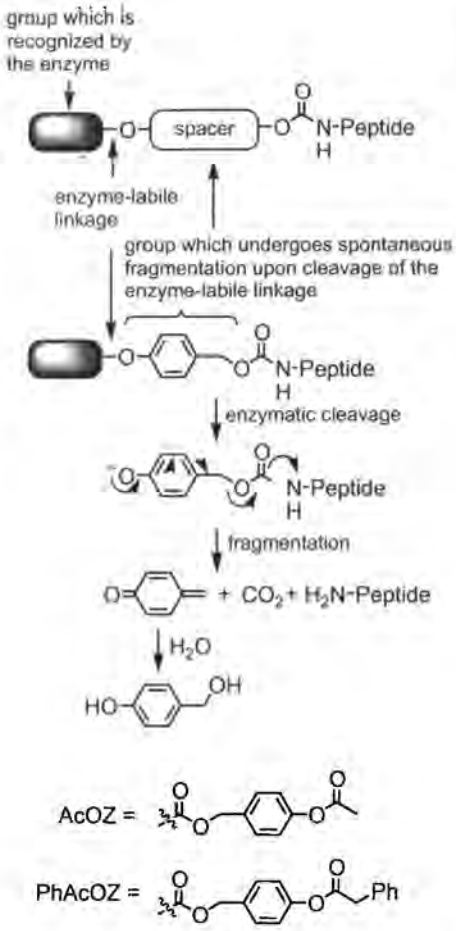


Figure 18-4. Principle of the spacer-based protecting groups AcOZ and PhAcOZ.

(Fig. 18-5)[62, 63]. During the synthesis the carbohydrate hydroxy functions are blocked by either benzyl ethers in the tetra-*O*-benzyl-D-glucopyranosyloxycarbonyl (BGloc) group or acetyl groups in the tetra-*O*-acetyl-D-glucopyranosyloxycarbonyl (AGloc) or the tetra-*O*-acetyl-β-D-galactopyranosyloxycarbonyl (AGaloc) protecting groups. The removal of these carbohydrate-based protecting groups proceeds via a two step process by removing the hydroxy blocking function in a first step followed by treatment with a glucosidase (AGloc, BGloc) or galactosidase (AGaloc), respectively. In the case of the acetyl derivatives AGloc and AGaloc a sequential two step process as well as a one-pot procedure were developed for the deprotection reaction, allowing for a convenient deprotection protocol as demonstrated for dipeptide **11** (Fig. 18-5)[62].

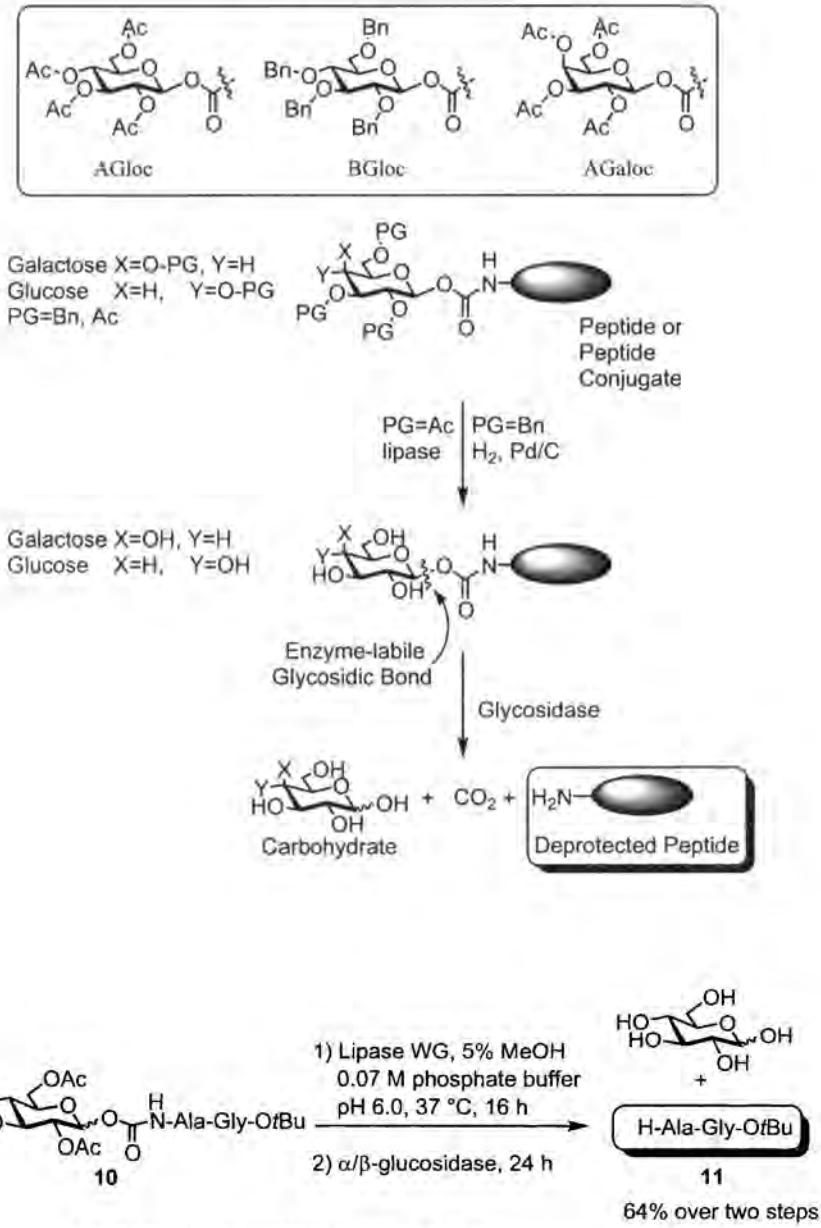


Figure 18-5. Carbohydrate-based urethane protecting groups.

18.2.3
Protection of the Side Chain Amino Group of Lysine

During chemical peptide syntheses and if trypsin is used for the construction of the peptide bonds or *N*-terminal deprotection, the side chain amino group of lysine generally has to be protected to prevent side reactions[13, 14]. This goal can be achieved enzymatically by applying the penicillin G acylase-catalyzed removal of the PhAc group (*vide supra*)[64]. Thus, the first application of the PhAc group in peptide chemistry was a synthesis of 1-deamino-Lys^8-vasopressin from the protected congener **9**, during which the lysine side chain was masked as the phenylacetamide (Fig. 18-3). After the peptide chain had been assembled and the disulfide bond was formed by oxidative cyclization, the PhAc group could be removed enzymatically in 74% yield without side reaction. A further interesting example which demonstrates that this technique can be applied advantageously to the synthesis of even larger peptides is found in the complete deprotection of $(PhAc)_3$porcine insuline (*vide supra*, Fig. 18-3)[27] and modified insuline fragments[65]. Since penicillin acylase is commercially available and devoid of peptidase activity[66], this method appears to be generally useful for the construction of lysine-containing oligopeptides.

In addition to the PhAc group, pyroglutamyl amides (Glp) were proposed as enzymatically removable blocking functions for the lysine side chain[23]. Their removal was achieved with pyroglutamate aminopeptidase from calf liver. Thus, all *N*-protecting groups were split off from the protected RNAse 1–10 fragment Glp-Lys(Glp)-Glu-Thr-Ala-Ala-Ala-Lys(Glp)-Phe-Glu-Arg-OH and from a model dipeptide. The general usefulness of this method remains to be demonstrated, however.

18.2.4
Protection of Amino Groups in β-Lactam Chemistry

The enzymatic removal of acyl groups plays an important role in the industrial production of semisynthetic penicillins and cephalosporins. To this end, penicillin G **12** (R = CH_2-Ph) and penicillin V **12** (R = CH_2-O-Ph), or the respective cephalosporins are first deacylated by means of penicillin acylases (Fig. 18-6)[67, 68]. The 6-aminopenicillanic acid and the 7-aminocephalosporanic acid thus obtained are subsequently acylated by non-enzymatic or enzymatic methods to give the semisynthetic antibiotics **13**.

The manufacture of therapeutically important cephalosporins from penicillin G and V includes a chemical ring expansion of the thiazolidine ring to a dihydrothiazine. In the course of this sequence the amino group remains protected as phenylacetyl or phenoxyacetyl amide, which is finally removed using penicillin G or V acylase. Of particular importance is the choice of a suitable protecting function for the COOH group. It must be stable during the ring expansion but removable without damaging the ceph-3-em nucleus. As an alternative to chemical methods, the use of the phenylacetoxymethylene ester was suggested for this purpose[41, 69]. It is easily introduced and is stable during the construction of the cephalosporin framework (Fig. 18-6). Together with the phenylacetamide the ester can eventually be

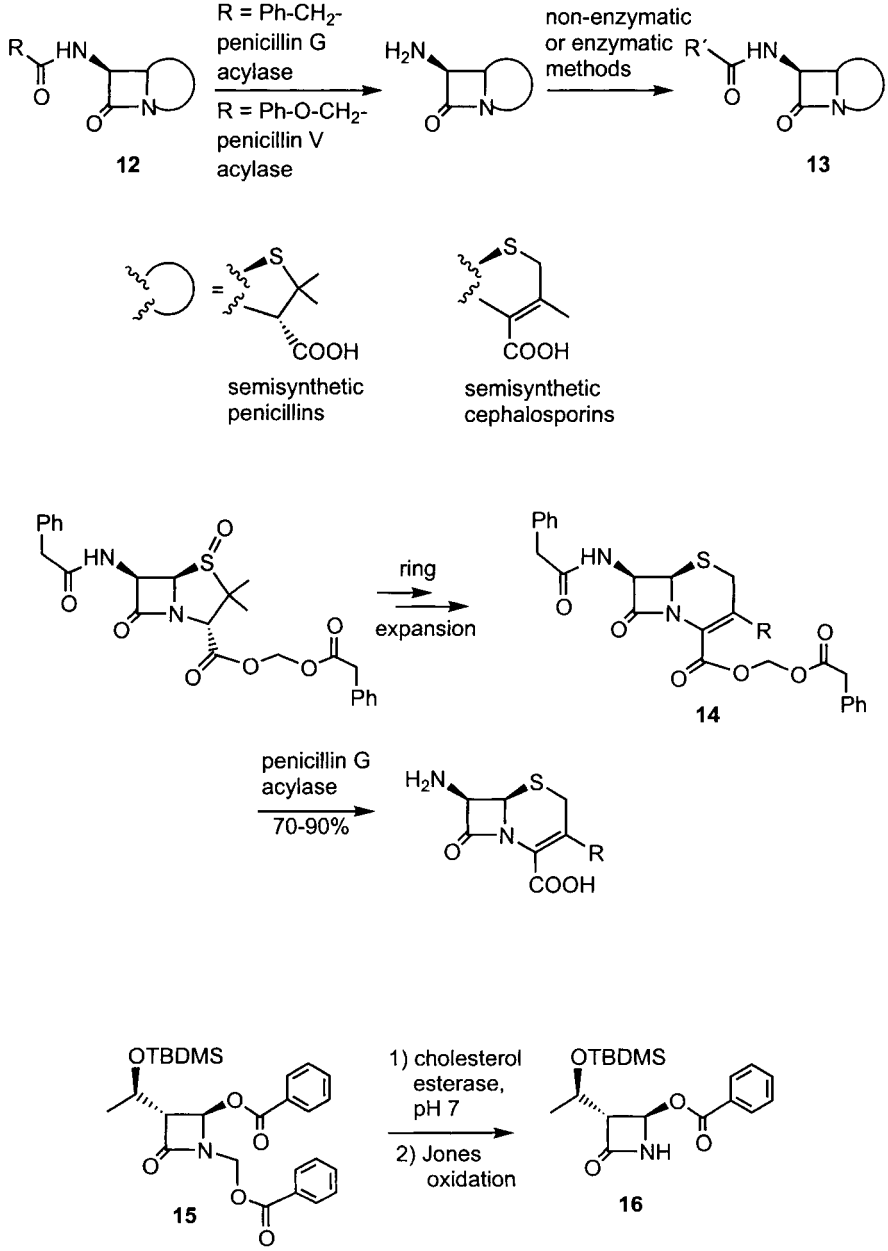


Figure 18-6. Enzymatic deprotection of amino- and carboxy groups in β-lactam chemistry.

removed in high yield from penicillin G and the cephalosporins **14** by penicillin G acylase. The formaldehyde formed in the deprotection is not harmful to the enzyme.

In a new approach to the well known versatile β-lactam building blocks, an enzymatic deprotection of an acylated methylol amide was applied with advantages (Fig. 18-6)[70]. Thus, the dibenzoate **15** was regioselectively saponified by cholesterol esterase at pH 7 giving rise to a monoacylated aminal. After Jones oxidation and subsequent loss of formaldehyde, the azetidinone **16** was obtained, which can be transformed into various enantiomerically pure penem and carbapenem building blocks.

As an alternative to the well established phenylacetyl group in β-lactam chemistry, recently a biocatalyzed procedure for the removal of phthalyl imide has been described (Fig. 18-2)[45, 71]. Its general usefulness remains to be demonstrated, however.

18.2.5
Protection of Amino Groups of Nucleobases

In general, the amino groups of the nucleobases adenine, guanine and cytosine in general must be protected during oligonucleotide synthesis to prevent undesired side reactions. To this end, they usually are converted into amides which are finally hydrolyzed under fairly basic conditions. If the amino functions are, however, masked as phenylacetamides, the protecting functions can be cleaved off by again employing penicillin G acylase (Fig. 18-7)[72–78]. The enzyme, for instance, selectively liberates the amino groups of the deoxynucleosides **17** without attacking the acetates in the carbohydrate parts and without damage to the acid-labile *N*-glycosidic bonds.

The biocatalyzed phenylacetyl removal can be carried out using both solubilized or immobilized substrates[77]. The latter methodology has been developed using controlled pore glass (CPG) as a solid support (Fig. 18-7).

18.3
Protection of Thiol Groups[4–6, 8, 12]

18.3.1
Protection of the Side Chain Thiol Group of Cysteine

The liberation of the β-mercapto group of cysteine was also achieved by means of the penicillin G acylase mediated hydrolysis of phenylacetamides[33–35]. To this end, the SH group was masked with the phenylacetamidomethyl (PhAcm) blocking function (Fig. 18-7). After penicillin acylase-catalyzed hydrolysis of the amide incorporated in the acylated thioaminal (see, e.g. **18**), a labile S-aminomethyl compound is formed which immediately liberates the desired thiol. This technique was for instance applied in a synthesis of glutathione which was isolated as the disulfide **19**. In a related glutathione synthesis the method was used for the simultaneous liberation of the SH- and the N-terminal amino function of glutamine[34, 35].

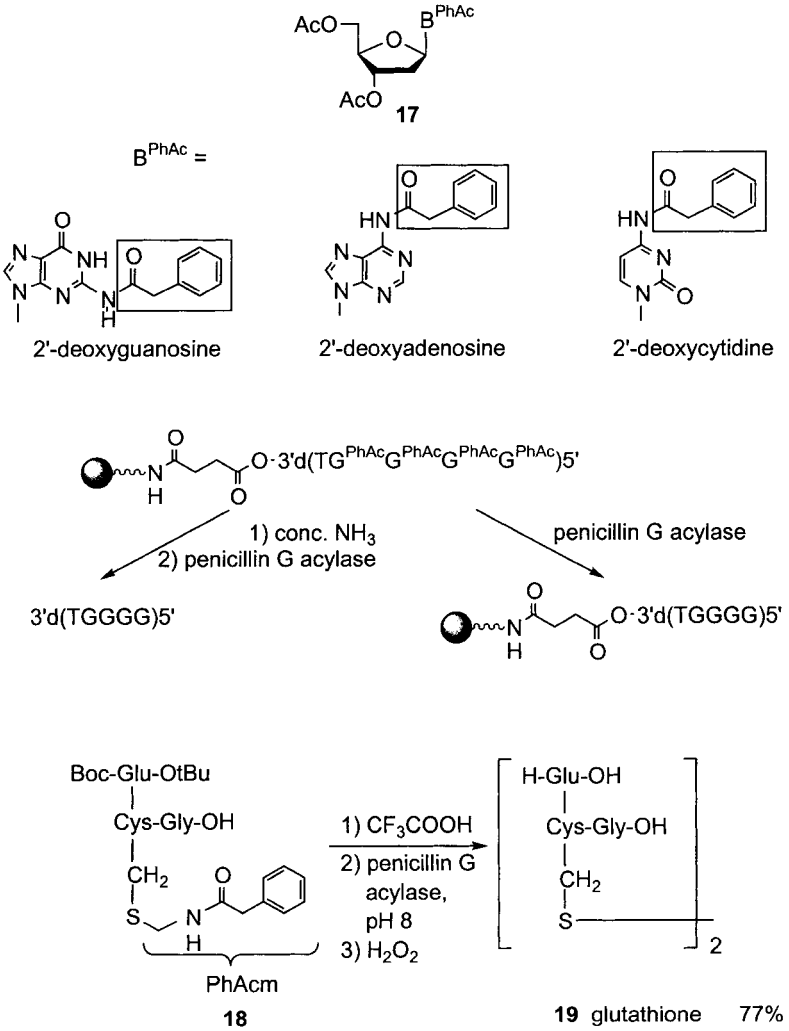


Figure 18-7. Enzymatic deprotection of the amino groups of nucleobases and the mercapto group of cysteine by means of penicillin G acylase. The shaded balls represent controlled pore glass (CPG).

18.4 Protection of Carboxy Groups[4–9, 12, 79]

18.4.1 C-Terminal Protection of Peptides

As in the enzymatic liberation of the *N*-terminus of peptides, initial attempts to achieve an enzyme-catalyzed deprotection of the corresponding carboxyl groups

concentrated on the use of the endopeptidases chymotrypsin[80–82], trypsin[81, 83, 84] and thermolysin[85], a protease obtained from *Bacillus thermoproteolyticus* which hydrolyzes peptide bonds on the amino side of hydrophobic amino acid residues (e.g. leucine, isoleucine, valine, phenylalanine). This latter biocatalyst enables the cleavage of the "supporting" tripeptide ester H-Leu-Gly-Gly-OEt from a protected undecapeptide to take place (pH 7, room temperature). The octapeptide thereby obtained was composed exclusively of hydrophilic amino acids. Owing to the broad substrate specificity of thermolysin and the resulting possibility of unspecific peptide hydrolysis this method can not be regarded as being generally applicable.

The exploitation of the esterase activities of chymotrypsin and trypsin opened routes to the hydrolysis of several peptide methyl, ethyl and *tert*-butyl esters at pH 6.4 to 8 and room temperature[80, 81]. The transformations are not only successful with peptides carrying the respective enzyme-specific amino acids at the *C*-terminus, but in several cases different amino acids were also tolerated at this position. However, severe drawbacks of this methodology are that numerous peptides are poor substrates or are not accepted at all. Moreover, a competitive cleavage of the peptide bonds occurs if the peptides contain trypsin- or chymotrypsin-labile sequences. Therefore, these proteases appear not to be generally useful for a safe *C*-terminal deprotection as well.

The disadvantages of using by the endopeptidases can be overcome by using carboxypeptidase Y from baker's yeast[25, 86, 87]. This serine-exopeptidase also has esterase activity and is characterized by quite different pH-optima for the peptidase and the esterase activity (pH >8.5). Even in the presence of various organic cosolvents the enzyme selectively removes the carboxy protecting groups from a variety of differently protected di- and oligopeptide methyl and ethyl esters[25, 87] without attacking the peptide bonds. An additional attractive feature is, that its esterase activity is restricted to α-esters, consequently β- and γ-esters of aspartic and glutamic acid, respectively, are not attacked. Carboxypeptidase Y was used advantageously for the stepwise *C*-terminal elongation of the peptide chain in aqueous solution employing a solubilizing poly(ethylene glycol) derived polymeric support as the *N*-terminal blocking group[86]. In a further remarkable synthesis which did not include the use of a polymeric *N*-protecting group, Met-enkephalin **20** was built up employing carboxypeptidase Y for *C*-terminal deprotection of intermediary generated peptide amides as well as for the formation of the peptide bonds (Fig. 18-8)[25].

The additional opportunity to hydrolyze selectively *C*-terminal peptide amides with carboxypeptidase Y is of particular interest if, as is demonstrated in the above mentioned example, enzymatic methods are applied to the formation of the peptide bonds, because amino acid amides are often the nucleophiles of choice in these biocatalyzed processes. For this purpose a peptide amidase from the flavedo of oranges shows very promising properties[88–90]. The enzyme is equipped with a broad substrate specificity and accepts Boc-, Trt-, Z- and Bz-protected and *N*-terminally unprotected peptide amides (Fig. 18-8). The *C*-terminal amides are saponified in high yields at pH 7.5 and 30 °C without affecting the *N*-terminal blocking groups or the peptide bonds. A noticeable advantage of this biocatalyst is

H–[Tyr]–Gly–Gly–[Phe]–Met–OH

20 methionine enkephalin

[ ] = C-terminally deprotected by enzymatic saponification of the peptide amide with carboxypeptidase Y;Tyr was N-terminally deprotected by removal or Bz-Arg with trypsin

$$\text{PG-peptide-NH}_2 \xrightarrow[\text{pH 7.5, 30°C}]{\text{amidase from the flavedo of oranges}} \text{PG-peptide-OH}$$

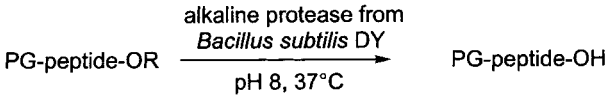
PG	peptide	conv. [%]
Bz	Tyr-Ser	100
Boc	Leu-Val	20
Trt	Gly-Leu-Val	100
Z	Gly-Gly-Leu	100

Figure 18-8. *C*-terminal deprotection of peptide amides by carboxypeptidase Y and an amidase from the flavedo of oranges.

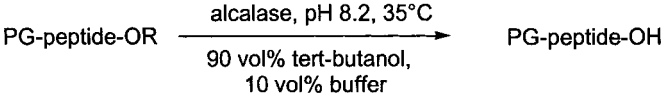
that *N*-deprotected amino acid amides, in contrast to the respective peptide amides, do not belong to its substrates. They can, therefore, be used as nucleophiles in peptide syntheses catalyzed by this enzyme, i.e. the formation of the peptide bond together with the subsequent *C*-terminal deprotection is achieved in a single step.

A further possibility for the enzymatic removal of *C*-terminal blocking groups is opened up by the application of enzymes which generally display a high esterase/protease ratio. Such a biocatalyst is the alkaline protease from *Bacillus subtilis DY* which shows similarities to Subtilisin Carlsberg. For this enzyme the ratio of esterase to protease activity is $>10^5$. It selectively removes methyl, ethyl and benzyl esters from a variety of Trt-, Z- and Boc-protected di- and tripeptides and a pentapeptide at pH 8 and 37 °C (Fig. 18-9)[91].

The *N*-terminal urethanes and the peptide linkages are left intact. A further protease which fulfills the requirements for a successfull application in peptide chemistry is alcalase, a serine endopeptidase from *Bacillus licheniformis* whose major component is subtilisin A (Subtilisin Carlsberg)[92–94]. It can advantageously be employed with advantage to selectively saponify peptide methyl and benzyl esters (Fig. 18-9). In a solvent system consisting of 90% *tert*-butanol and 10% buffer (pH 8.2) even highly hydrophobic and in aqueous solution insoluble Fmoc peptides were accepted as substrates and deprotected at the C-terminus without any disturbing side reactions. A selective classical alkaline saponification of methyl esters would be impossible due to the base-sensitivity of the Fmoc group.



PG	peptide	R	yield [%]
Z	Tyr(tBu)-Glu-Leu	Me	93
Boc	Leu-Glu-Val	Bzl	85
Trt	Ala-Glu-Asp-Leu-Glu	Bzl	80

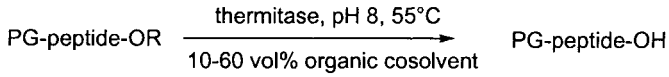


PG	peptide	R	yield [%]
Fmoc	Ala-Val-Ile	Me	85
Fmoc	Asn-Phe	Bzl	90
Boc	Met-Leu-Phe	Me	80
Z	Met-Asp(OMe)-Phe	Me	90

Figure 18-9. *C*-terminal deprotection of peptide esters by the alkaline protease from *Bacillus subtilis DY* and alcalase.

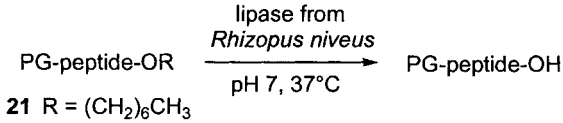
A very promising and unusually stable biocatalyst is thermitase, a thermostable extracellular serine protease from the thermophilic microorganism *Thermoactinomyces vulgaris* whose esterase/protease ratio amounts to >1000 : 1. The enzyme shows a broad amino acid side chain specificity and cleaves methyl, ethyl, benzyl, methoxybenzyl and *tert*-butyl esters from a variety of Nps-, Boc-, Bpoc- and Z-protected di- and oligopeptides in high yields at pH 8 and 35–55 °C (Fig. 18-10)[33, 34, 95–97]. In addition, it is specific for the α-carboxy groups of Asp and Glu. To enhance the solubility of the substrates, furthermore, up to 50 vol% of organic cosolvents such as DMF and DMSO may be added which also serve to reduce the remaining peptidase activity to a negligible amount[34, 97].

In the discussion of the protease-catalyzed cleavage of the *N*-terminal protecting groups it has already been pointed out that the use of biocatalysts belonging to this class of enzymes in general, i.e. also for the *C*-terminal deblocking, may lead to an undesired hydrolysis of peptide bonds. In particular, this has to be expected if the respective ester or amide to be hydrolyzed turns out to be only a poor substrate, which is only attacked slowly, an experience not uncommon if unnatural substrates are subjected to enzyme mediated transformations. This undesired possibility would, however, be overcome if enzymes were used which were not able to split amides at all. This principle has been realized in the development of the heptyl



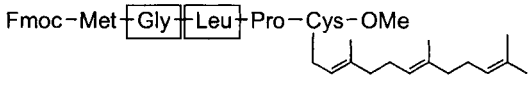
PG	peptide	R	yield [%]
Z	Leu-Val-Glu(tBu)-Ala	Me	92
Boc	Pro-Gly	Me	73
Bpoc	Tyr(tBu)-Glu-Leu	Me	55
Nps	Ser(Bzl)-His(Dnp)-Leu-Val-Glu(tBu)-Ala	Me	90

Figure 18-10. *C*-terminal deprotection of peptide esters by thermitase.



21 R = $(CH_2)_6CH_3$
22 R = $(CH_2)_2Br$

PG	peptide	R	yield [%]
Boc	Ser-Thr	Hep	95
Z	Thr-Ala	Hep	85
Aloc	Met-Gly	Hep	90
Z	Ser-Phe	EtBr	84
Boc	Val-Ala	EtBr	95



23 C-terminal pentapeptide of the N-Ras protein

□ = C-terminally deprotected by employing lipase from *Rhizopus niveus*

Figure 18-11. *C*-terminal deprotection of peptide esters by lipase from *Rhizopus niveus*.

(Hep),[4–9, 31, 32, 98–100] the 2-bromoethyl (EtBr)[4–6, 31, 32, 101] and the *p*-nitrobenzyl (PNB) esters[102] as carboxy protecting groups for peptide synthesis which can be enzymatically removed by means of lipases or esterases, respectively (Fig. 18-11).

The Hep-esters proved to be chemically stable during the removal of the *N*-terminal Z-, Boc- and the Aloc-group from the dipeptides **21**. The selective removal of the Hep-esters was achieved by a lipase-catalyzed hydrolysis. From several enzymes investigated, a biocatalyst isolated from the fungus *Rhizopus niveus* was superior to the others with respect to substrate tolerance and reaction rate. The enzyme accepts a variety of Boc-, Z- and Aloc-protected dipeptide Hep-esters as substrates and hydrolyzes the ester functions in high yields at pH 7 and 37 °C

without damaging the urethane protecting groups and the amide bonds (Fig. 18-11)[98, 99]. Z- and Boc-dipeptide-2-bromoethyl esters **22** are also attacked, at a comparable or in some cases even higher rate. In the presence of either one of the enzyme-labile protecting groups the *N*- and *C*-terminal amino acid can be varied considerably. With increasing steric bulk and lipophilicity of the amino acids, in particular the *C*-terminal one, the rate of the enzymatic reactions decreases. If the *C*-terminal amino acid is proline, the enzymatic reaction does not take place. The lipase-mediated deprotection of peptides was for instance successfully applied in the construction of the *C*-terminal pentapeptide methyl ester **23** of the N-Ras-protein, which is localized in the plasma membrane and which plays a vital role in cellular signal transduction (Fig. 18-11)[103].

The use of lipases for the removal of protecting groups from peptides in addition to the absence of protease activity has several advantages. Various enzymes belonging to this class and stemming from different natural sources (including mammals, bacteria, fungi and thermophilic organisms) are commercially available and fairly inexpensive. This variety provides the opportunity of replacing a chosen biocatalyst by a better one if a particular substrate is only attacked slowly (*vide infra*). The lipases are not specific for L-amino acids but also tolerate the presence of the D-enantiomer[104]. A noticeable feature is that, in contrast to proteases and esterases, they operate at the interface between water and organic solvents[105]. This is particularly important if longer peptides, which are composed of hydrophobic amino acids and/or carrying side chain protecting groups, and that do not dissolve well in the aqueous systems, have to be constructed.

The full capacity of the lipase mediated technique for *C*-terminal deprotection was demonstrated by the synthesis of complex base-labile phosphopeptides[44] and *O*-glycopeptides, which are sensitive to both acids and bases[106, 107]. To this end, e.g. the serine glycoside **24** was selectively deprotected at the *C*-terminus by lipase from the fungus *Mucor javanicus* (Fig. 18-12).

The carboxylic acid **25** liberated thereby was then coupled with an *N*-terminally deprotected glycodipeptide and after subsequent enzyme-mediated deprotection the glycotripeptide carboxylic acid **26** was obtained in high yield. This compound was finally condensed with a tripeptide to give the complex diglycohexapeptide **27**, which carries the characteristic linkage region of a tumor-associated glycoprotein antigen found on the surface of human breast cancer cells. In the course of these enzymatic transformations, the *N*-terminal urethanes, the peptide bonds, the acid- and base-labile glycosidic linkages and the acetyl protecting groups, being sensitive to bases, were not attacked. In these cases lipase from *Rhizopus niveus* which was the enzyme of choice for simple peptides only attacked the substrates slowly, so that a different biocatalyst had to be used. This demonstrates the above mentioned advantage of being able to apply several catalytic proteins of comparable activity but different substrate tolerance for the solution of a given synthetic problem.

The viability and the wide applicability of the principle of using enzymes for the removal of individual protecting groups from complex multifunctional compounds such as lipo- and glycopeptides is furthermore proven by the finding that proteases can also be used for this purpose. Thus, by means of thermitase-catalysis the *C*-

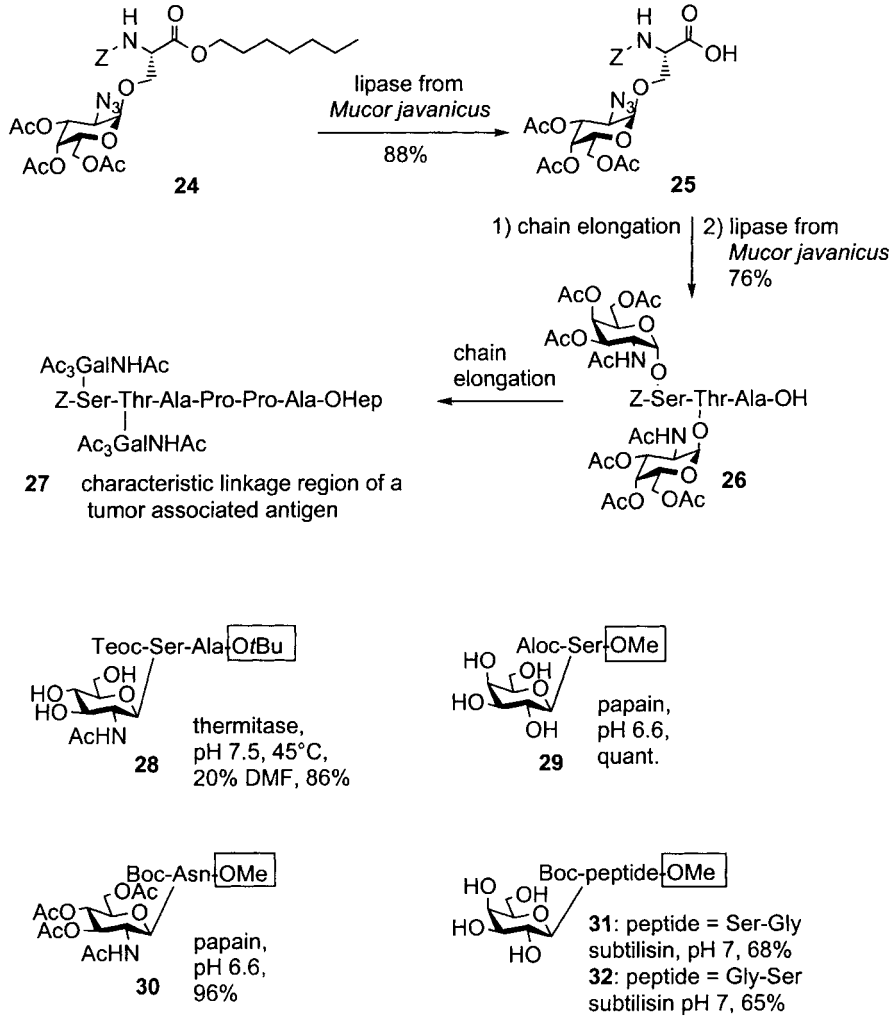
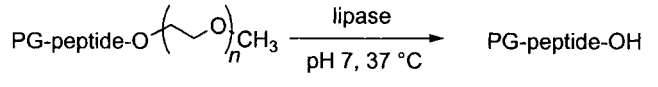


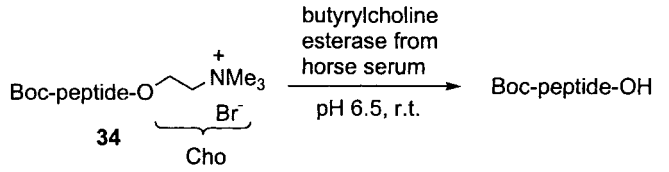
Figure 18-12. Construction of acid- and base labile glycopeptides via enzyme-mediated C-terminal deprotection.

terminal *tert*-butyl ester was removed from the glycopeptide **28** (Fig. 18-12)[34, 108]. In a different study, this enzyme was also used for the cleavage of methyl and *p*-nitrobenzyl esters[109]. From the serine glycoside **29**[110, 111] and from the asparagine conjugate **30**[112] the methyl esters could be cleaved off without disturbing side reactions by using papain as the biocatalyst. Similarly, the liberation of the *C*-terminal carboxy group of the glycosylated dipeptides **31** and **32** was achieved by means of subtilisin-catalyzed hydrolysis[113]. However, in these cases papain could not be used since this protease preferably cleaved the peptide bonds. This example again highlights the danger associated with the use of a protease for the removal of protecting groups from peptides.

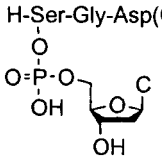
A problem arising regularly in the enzymatic deprotection is the poor solubility of the fully blocked peptides in the required aqueous media, resulting in a limited accessibility of the substrates to the enzymes. To overcome this difficulty, in many cases solubilizing organic cosolvents are added, however, a more general and viable approach consists of the introduction of solubilizing protecting groups, e. g. in the enzyme-mediated formation of peptide bonds (see Chapter B 2.5)[114]. An enzymatically removable solubilizing ester protecting group could be found in the ethylene glycol derived esters such as the methoxyethyl (ME) esters[78, 115], and the methox-



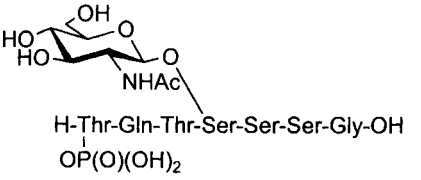
33 n=1: methoxyethyl (ME)
n=2: methoxyethoxyethyl (MEE)



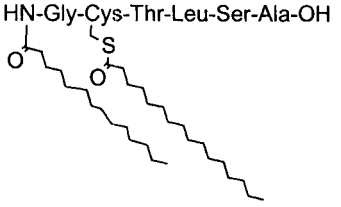
34



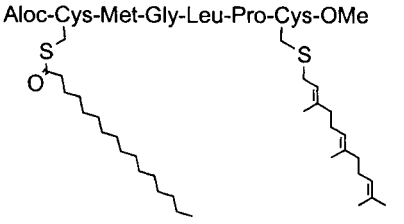
adenovirus 2 nucleoprotein



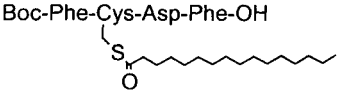
serum response factor (SRF)



$G_{\alpha 0}$-protein



N-Ras protein



human Y_1 receptor

Figure 18-13. Use of hydrophilic esters as solubilizing enzymatically removable protecting groups for the synthesis of characteristic protein fragments.

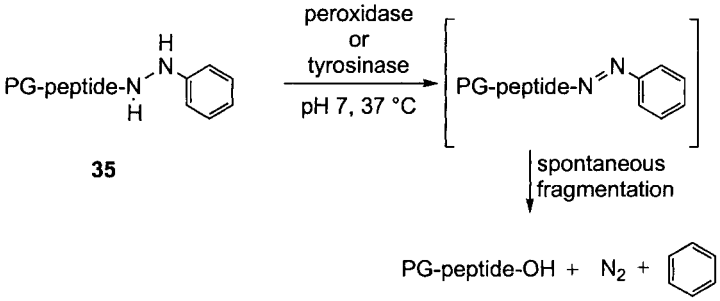


Figure 18-14. Phenylhydrazide as a carboxy protecting group.

yethoxyethyl (MEE) esters[78, 115–117] and in the choline esters (Fig. 18-13)[58, 59, 76, 78, 118–121]. The ME and MEE esters serve both as hydrophilic analogues of the heptyl esters discussed above and can therefore be removed by the same biocatalysts such as the lipase from *Mucor javanicus*. Their increased solubility in aqueous media has been used successfully in the synthesis of small peptides and peptide conjugates including glyco-[115–117] and nucleopeptides[78].

Similarly, the respective dipeptide choline esters **34** are readily soluble in purely aqueous media (i. e. without added cosolvent) and are converted into the corresponding carboxylic acids under the mildest conditions, and without side attack on the peptide bonds and the *N*-terminal urethanes, by means of the commercially available butyrylcholine esterase from horse serum. The increased hydrophilicity of peptide choline esters was used advantageously used for the synthesis of peptides and very sensitive peptide conjugates such as lipidated peptides[118–121], phosphorylated and glycosylated peptides[58, 59] and nucleopeptides (Fig. 18-13)[76, 78].

Recently, phenylhydrazide has been introduced as an enzyme-labile carboxy protecting group[122, 123]. This protecting group can be removed by mild enzymatic oxidation using a peroxidase[122, 123] or mushroom tyrosinase[124] (Fig. 18-14).

18.4.2
Protection of the Side Chain Groups of Glutamic and Aspartic Acid

The stepwise removal of arginine methyl ester by proteases has been investigated as a possibility for the enzymatic deprotection of the side chain carboxylate groups of the aminodicarboxylic acids aspartic acid (Asp) and glutamic acid (Glu). To this end, Z-Asp(ArgOMe)-NH_2 and Z-Glu(ArgOMe)-NH_2 were converted into Z-Asp(OH)-NH_2 and Z-Glu(OH)-NH_2 by subsequent treatment with trypsin, which hydrolyzes the arginine methyl esters, and with porcine pancreatic carboxypeptidase B, which splits off the arginines[125]. Since the second step is slow and requires high concentrations of the carboxypeptidase, this method can, most probably, not be applied routinely in peptide synthesis because it introduces too much of a danger of competitive side reactions.

However, enzymatic transformations have proved to be useful for the synthesis of selectively functionalized aspartic and glutamic acid derivatives. For instance,

alcalase selectively hydrolyzes the α-benzyl esters of H-Asp(Bzl)-OBzl and H-Glu(Bzl)-OBzl in 82% and 85% yield, respectively, on a decagramm scale[126]. Similarly, aspartyl- and glutamylpeptides can be deprotected selectively at the *C*-terminus by this enzyme, however, in these cases an undesirable attack on the peptide bonds may occur[127]. In addition, Z-Asp(OAll)-OAll is converted into Z-Asp(OAll)-OH in quantitative yield by papain[128]. Also a lipase from *Candida cylindracea* is able to differentiate between the two carboxylic acid groups of glutamic acid. From the respective di-cyclopentyl ester it preferably (ratio 20 : 1) removes the γ-ester in 90% yield[129]. In addition, the enzyme thermitase and the alkaline protease from *Bacillus subtilis* (*vide supra*) also have great potential for the selective manipulation of dicarboxylic amino acids.

The examples given in Sections 18.2 to 18.4 demonstrate that the selective deprotection of peptides can be achieved advantageously by making use of enzymatic reactions. In the light of the increasing number of available biocatalysts it appears that in the near future a host of new and superior enzymatically removable blocking groups for the synthesis of peptides will be developed. However, these techniques will definitely not be used for the preparation of simple small peptides in the laboratory. Most probably they will be applied to the synthesis of sensitive polyfunctional compounds and long oligopeptides, the construction of which is cumbersome by standard chemical methods. Furthermore, they offer significant advantages if a technical process for the manufacturing of a given peptide has to be developed. Finally, together with the recently developed methods for the biocatalyzed formation of peptide bonds (see Chapter 12.5)[130], enzymatic protecting group techniques could prove to be the tools of choice for the construction of peptides in aqueous solution, the practical development of which has been tried for several decades[131,132].

18.5
Protection of Hydroxy Groups[4–9, 133–136]

Mono- and oligosaccharides, alkyl- and arylglycosides and various other glycoconjugates generally include a multitude of hydroxyl groups of comparable chemical reactivity. Also, the synthesis of oligonucleotides and nucleosides, ß-lactams, alkaloids, steroids and peptides often requires the selective protection of one or more alcoholic functions. Consequently, for the directed construction of polyhydroxy compounds these functional groups have to be manipulated selectively, in general making cumbersome protection and deprotection steps necessary. Although numerous chemical techniques are available to mask or to liberate hydroxyl groups,[1–3] the development of enzymatic methods for this purpose has been progressing steadily and appears to complement the arsenal of classical tools. In addition, the enzymatic protection of hydroxy goups (and vice versa of carboxy groups) in racemic compounds as well as their enzyme-catalyzed deprotection has been used extensively for the separation of enantiomeric alcohols and carboxylic acids (see Chapter 11).

18.5.1
Protection of Monosaccharides[133, 137]

The selective protection and deprotection of carbohydrates can be achieved with various classical chemical techniques[1–3, 138–140]. In addition, however, owing to the synthetic challenge the multifunctional carbohydrates pose, enzymatic techniques for the introduction of blocking groups into sugars and/or their subsequent removal offer further, different opportunities.

The enzymatic acylation of sugars in aqueous solution has been reported but gives low yields as the equilibrium for the reaction favors hydrolysis. However, enzymatic acylation in dry organic solvents has shown substantial success. While direct enzymatic esterification of alcohols with acids is often not practical, good to excellent yields have been obtained using transesterification techniques (Table 18-1). The displacement of the equilibrium toward products has been accomplished by using an excess of the acyl donor and by using activated, irreversible acyl donors such as trihaloethyl esters[141], enol esters[142], acid anhydrides or oxime esters[134, 136]. In particular, the enol esters have the advantage that the liberated enol tautomerizes to a ketone or an aldehyde, thereby shifting the equilibrium toward the desired products and consequently giving higher yields. This technology, however, is not restricted to carboxylic acid derivatives being the acyl donor. Organic carbonates[143], either activated as the vinyl[144] or, even better, as an oxime[145] derivative, allow for the enzyme-catalyzed synthesis of carbonates such as the methoxycarbonyl, the benzyloxycarbonyl (Z) and the allyloxycarbonyl (Aloc) carbonate. The last two examples can later be removed by non-enzymatic means.

The high polarity of sugars and their derivatives requires that polar solvents be used to dissolve them. Solvents found to be suitable include pyridine, DMSO, DMF and dimethylacetamide. However, these solvents also often inactivate enzymes, although some enzymes, for instance the lipases from the porcine pancreas (PPL), from *Candida antarctica* (CAL), from *Candida cylindracea* (CCL, later renamed *Candida rugosa*) and the lipase from *Pseudomonas cepacia* (PSL) as well as the proteases subtilisin and proleather, maintain their inherent acitvity[146]. A less polar solvent such as THF allows the use of a broader variety of lipases, but does not dissolve unmodified pyranoses. Nevertheless, it should be noted that even glucose suspended in THF has been successfully acylated by using lipase of *Candida antarctica*[147].

To remain active in an organic solvent, the enzyme must contain a small amount of water which is required for maintaining the correct protein structure. In the absence of this essential water, highly polar compounds such as carbohydrates form excessively tight enzyme-product complexes. This inhibits association and dissociation of substrates and products from the active site and thus slows down the reaction. Accordingly, the addition of drying agents such as zeolite CaA not only influences activity of the the biocatalyst but also its selectivity. For instance, the acylation of 1-*O*-methyl β-D-glycopyranoside **49** catalyzed by lipase SP 435 (an immobilized lipase from *Candida antarctica*) in ethyl butanote as the solvent and acyl donor led to acylation predominantly in the 6-position[148, 149]. If zeolite CaA was added, a

mixture of 2,6- and 3,6-bisacylated pyranosides (95 : 5) was formed. In the presence of zeolite CaA and *tert*-butanol as a cosolvent, again monoacylation in the 6-position was observed.

Alternatively, precipitation of the enzyme from aqueous solution at its optimum pH prior to its use in an organic solvent has also been reported to increase the enzyme's activity greatly.

The results of enzymatic acylation of several pyranose and furanose sugars are shown in Table 18-1. Other lipophilic carbohydrate derivatives such as alkyl glycosides also display a higher solubility in less polar organic solvents, in which most lipases tend to be more stable than in polar solvents.

A further interesting finding is that heat stable lipases are capable of transferring long-chain fatty acids to the 6-hydroxy group of ethyl glucoside on a kilogram-scale, utilizing the molten fatty acids themselves as solvents[171]. On a somewhat smaller scale, the acylation of glucose has also been carried out using only a minute amount of solvent[172] or in supercritical CO_2[173, 174].

The regioselectivity observed in the acylation of underivatized pyranoses in principle parallels that recorded for the classical chemical introduction of acyl groups into carbohydrates. However, if the 6-OH groups are protected first or deoxygenated, in the corresponding enzymatic reactions selectivities are observed which can not be realized with classical chemical methods. By careful choice of solvent and lipase, it is possible to modifiy selectively a number of C6 protected pyranoses at the secondary hydroxy groups (Table 18-2).

By combination of enzymatic with non-enzymatic protection group chemistry, carbohydrates can be selectively modified in the primary and secondary hydroxy positions. To demonstrate this versatility, the straightforward synthesis of differently mono-acylated glucose derivatives is described in Fig. 18-15. For instance, 6-*O*-butyrylated glucose **66a** (R = n-butanoyl; prepared enzymatically, see Table 18-1) is converted into the 3,6-dibutanoate **93** by lipase from *Chromobacterium viscosum* (CVL) or from *Aspergillus niger* (ANL). The 2,6-dibutanoate **94** can conveniently be built up with the lipase from porcine pancreas (PPL; Fig. 18-15)[164]. Similar observations were reported for n-octylglucoside, but for the corresponding galactose- and mannose 6-esters the selectivity was lower. In contrast, the chemical butyrylation of glucose derivative **66a** with the acid anhydride in pyridine gave a complex mixture of various diesters without any significant regiodiscrimination. The enzymatic approach was also used to convert the 6-*O*-tritylglucose **66b** (R = Trt) into the 3-butanoate **95** by a chemoenzymatic approach with lipase from *Chromobacterium viscosum* (CVL), and the 6-*tert*-butyl-diphenylsilylated glucose **66c** (R = TBDPS) could be acylated exclusively at the 2-position when employing lipase from *Candida cylindracea* (CCL)[164]. From the disubstituted glucoses obtained by the enzyme-catalyzed reactions, the protecting functions in the 6-position could be split off chemically or enzymatically, thus making the glucose esters **95** and **96** carrying a single acyl group in the 2- or the 3-position available in a convenient way (Fig. 18-15).

The monoacylated saccharides used in these studies dissolve in several organic solvents, of which tetrahydrofuran and methylenedichloride were found to be

Table 18-1. Selective acylation of the primary hydroxy group in monosaccharides.

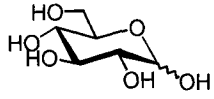
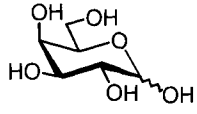
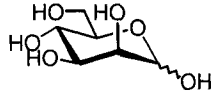
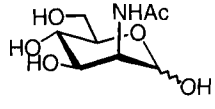
Compound No.	Structure	Enzyme[a]	Solvent	Acyl Donor	Position	Yield (%)	Ref.
36		PPL	pyridine	$RCO_2CH_2CCl_3$	6	19–35	[141]
		CAL	dioxane	$ROCO_2N{=}CMe_2$	6	15–72	[145]
		CAL	THF	$RCO_2CH{=}CH_2$	6		[147]
		PSL	pyridine	$MeCO_2CH_2CCl_3$	6	79	[146]
		PSL	pyridine	$EtCO_2CH_2CCl_3$	6	29	[146]
		proleather	pyridine	$PhCO_2CH_2CCl_3$	6	33	[146]
		subtilisin	DMF	$PrCO_2CH_2CCl_3$	6	60	[150]
		subtilisin	pyridine	$PrCO_2CH_2CCl_3$	6	64	[150]
		optimase M-440	pyridine	Boc-Phe-OCH_2CF_3	6		[151]
37		PPL	pyridine	$MeCO_2CH_2CCl_3$	6	57	[141]
		PSL	pyridine	$RCO_2N{=}CMe_2$	6	70–85	[152]
		CAL	dioxane	$ROCO_2N{=}CMe_2$	6	43–68	[145]
38		PPL	pyridine	$MeCO_2CH_2CCl_3$	6	36	[141]
		CCL	benzene/pyridine 2:1	$MeCO_2CH{=}CH_2$	6		[142]
		PSL	pyridine	$RCO_2N{=}CMe_2$	6	65–80	[152]
		CAL	dioxane	$ROCO_2N{=}CMe_2$	6	44–53	[145]
		protease N	DMF	$MeCO_2C(Me){=}CH_2$	6	40	[153]
39		CCL	benzene/pyridine 2:1	$MeCO_2C(Me){=}CH_2$	6		[142]
		protease N	DMF	$MeCO_2C(Me){=}CH_2$	6	73	[153]
		subtilisin 8399	DMF	$MeCO_2CH{=}CH_2$	6	92	[154]
		subtilisin BNP'	97% DMF	Boc-Gly-OCH_2CN	6	65	[155]
40		CAL	pyridine	$RCO_2N{=}CMe_2$	6	45–83	[156]
		PSL	dioxane	$RCO_2N{=}CMe_2$	6	50–72	[156]

Table 18-1. (cont.).

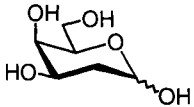
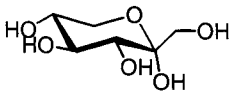
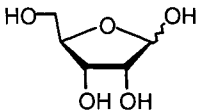
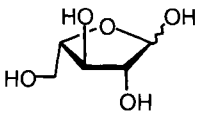
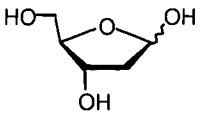
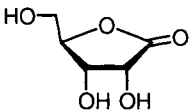
Compound No.	Structure	Enzyme[a]	Solvent	Acyl Donor	Position	Yield (%)	Ref.
41		CAL	pyridine	$RCO_2N{=}CMe_2$	6	57–81	[156]
		PSL	dioxane	$RCO_2N{=}CMe_2$	6	47–62	[156]
42		PSL	pyridine	$RCO_2N{=}CMe_2$	1	68–86	[152]
43		CAL	pyridine	$RCO_2N{=}CMe_2$	5	50–64	[152]
		CAL	dioxane	$ROCO_2N{=}CMe_2$	5	37–52	[145]
		CAL	THF	Pr_2O	5		[157,158]
44		CAL	pyridine	$RCO_2N{=}CMe_2$	5	45–70	[152]
		CAL	dioxane	$ROCO_2N{=}CMe_2$	5	38–49	[145]
45		CAL	THF	Pr_2O	5		[157,158]
46		PPL	pyridine	$C_{11}H_{23}CO_2CH_2CCl_3$	5	40	[159]

Table 18-1. (cont.).

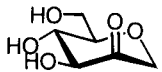
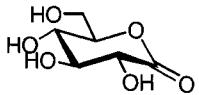
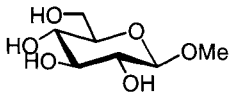
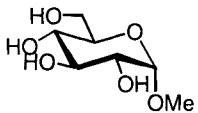
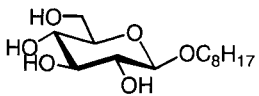
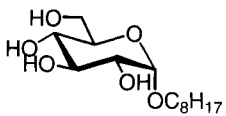
Compound No.	Structure	Enzyme[a]	Solvent	Acyl Donor	Position	Yield (%)	Ref.
47a		CAL	acetone/pyridine 3:1	$C_{11}H_{23}CO_2H$	6	67	[160]
47b		CAL	*t*BuOH	$C_{11}H_{23}CO_2Et$	6	51	[161]
48		CCL	benzene/pyridine 2:1	$MeCO_2CH{=}CH_2$	6		[142]
		CAL	THF/pyridine (4:1)	$MeCO_2CH{=}CH_2$	3,6		[162]
		CAL	$PrCO_2Et$/*t*BuOH (1:1)	$PrCO_2Et$	6		[148,149]
49		CAL	$CH_2{=}CHCO_2Et$/ *t*BuOH (1:1)	$CH_2{=}CHCO_2Et$	6		[149]
		CAL	THF/pyridine (4:1)	$MeCO_2CH{=}CH_2$	6		[162]
50		CAL	THF	$MeCO_2CH{=}CH_2$	6		[162]
		CAL	$PrCO_2Et$/*t*BuOH (1:1)	$PrCO_2Et$	6		[148,149]
		CAL	*t*BuOH	$Ph(CH_2)_3CO_2H$	6	52	[163]
		CVL	THF	$PrCO_2CH_2CCl_3$	6; 3,6 (1:1)		[164]
		ANL	THF	$PrCO_2CH_2CCl_3$	6; 3,6 (10:1)		[164]
51		CAL	$CH_2{=}CHCO_2Et$	$CH_2{=}CHCO_2Et$	6		[149]

Table 18-1. (cont.).

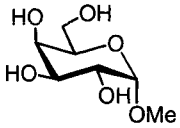
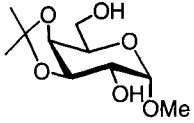
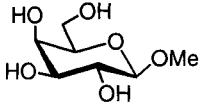
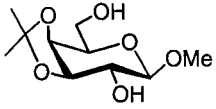
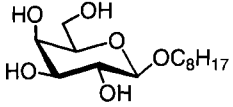
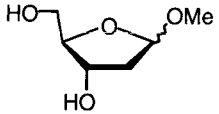
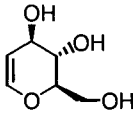
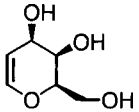
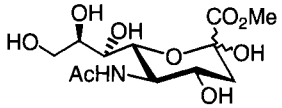
Compound No.	Structure	Enzyme[a]	Solvent	Acyl Donor	Position	Yield (%)	Ref.
52		PPL	pyridine	$PrCO_2CH_2CF_3$	6	79	[165]
		CAL	THF/pyridine (4:1)	$MeCO_2CH{=}CH_2$	6; 3,6 (3:1)		[162]
		CAL	$CH_2{=}CHCO_2Et$/ *t*BuOH (1:1)	$CH_2{=}CHCO_2Et$	2,6		[148,149]
53		PSL	$MeCO_2CH{=}CH_2$/THF	$MeCO_2CH{=}CH_2$	6	93	[166]
54		CAL	THF/pyridine (4:1)	$MeCO_2CH{=}CH_2$	6; 2,6; 3,6 (1:1.3:1.8)		[162]
		CAL	$CH_2{=}CHCO_2Et$/ *t*BuOH (1:1)	$CH_2{=}CHCO_2Et$	6; 2,6; 3,6 (2:1:1)		[148,149]
55		PSL	$MeCO_2CH{=}CH_2$/THF	$MeCO_2CH{=}CH_2$	6	90	[166]
56		PSL	$MeCO_2CH{=}CH_2$	$MeCO_2CH{=}CH_2$	6	75	[167]

Table 18-1. (cont.).

Compound No.	Structure	Enzyme[a]	Solvent	Acyl Donor	Position	Yield (%)	Ref.
57		PSL	$MeCO_2CH{=}CH_2$/THF	$MeCO_2CH{=}CH_2$	6	94	[166]
58		PPL	pyridine	$PrCO_2CH_2CF_3$	6	81	[165]
59		PPL	THF	$MeCO_2CH_2CF_3$	5	77	[168]
60		PPL	THF	$MeCO_2CH_2CF_3$	5	77	[168]
61		PPL	THF	$MeCO_2CH_2CF_3$	5	84	[168]

Table 18-1. (cont.).

Compound No.	Structure	Enzyme[a]	Solvent	Acyl Donor	Position	Yield (%)	Ref.
62		PPL	THF	$MeCO_2CH_2CF_3$	5 3	39 17	[168]
63		CCL	EtOAc	$MeCO_2CH{=}CH_2$	6	90	[169]
64		CCL	EtOAc	$MeCO_2CH{=}CH_2$	6	93	[169]
65		CCL	THF	$MeCO_2CH{=}CH_2$	9	60	[170]

a Many enzymes were usually screened for activity, only the best results are listed. CAL: *Candida antarctica* lipase; CCL: lipase from *Candida cylindracea* (later renamed *Candida rugosa*); PPL: porcine pancreas lipase; PSL: *Pseudomonas cepacia* lipase.

Table 18-2. Selective acylation of secondary hydroxy groups in monosaccharides.

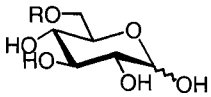
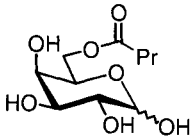
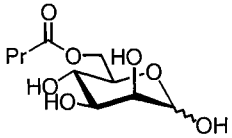
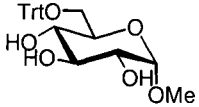
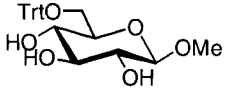
Compound No.	Structure	Enzyme[a]	Solvent	Acyl Donor	Position	Yield (%)	Ref.
66	 **a**: R=butyryl **b**: R=trityl **c**: R=TBDPS	ANL	THF	$PrCO_2CH_2CCl_3$	3 (**66a**)		[164]
		CVL	THF	$PrCO_2CH_2CCl_3$	3 (**66a**)	80	[164]
		PPL	THF	$PrCO_2CH_2CCl_3$	2 (**66a**)	51	[164]
		CVL	THF	$PrCO_2CH_2CCl_3$	3 (**66b**)	88	[164]
		PFL	$MeCO_2CH{=}CH_2$	$MeCO_2CH{=}CH_2$	2 (**66b**)		[175]
		CCL	CH_2Cl_2	$PrCO_2CH_2CCl_3$	2 (**66c**)	45	[164]
67		CVL	THF	$PrCO_2CH_2CCl_3$	2	20	[164]
					3	31	
68		CVL	THF	$PrCO_2CH_2CCl_3$	2	13	[164]
					3	52	
69		lipase from *Mucor miehei*	$MeCO_2CH{=}CH_2$	$MeCO_2CH{=}CH_2$	2		[175]
70		PFL	$MeCO_2CH{=}CH_2$	$MeCO_2CH{=}CH_2$	3		[175]
		lipase from ft2Mucor miehei	$MeCO_2CH{=}CH_2$	$MeCO_2CH{=}CH_2$	2		[175]

Table 18-2. (cont.).

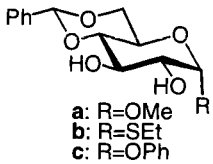
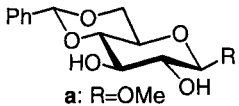
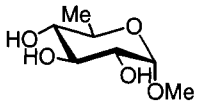
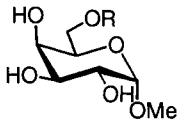
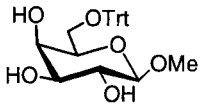
Compound No. Structure	Enzyme[a]	Solvent	Acyl Donor	Position	Yield (%)	Ref.
71  a: R=OMe b: R=SEt c: R=OPh	PSL	$RCO_2CH_2CF_3$/THF	$RCO_2CH_2CF_3$	2 (**71a**)		[176]
		$RCO_2CH{=}CH_2$/THF	$RCO_2CH{=}CH_2$	2 (**71a**)		
	PSL	$MeCO_2CH{=}CH_2$	$MeCO_2CH{=}CH_2$	2 (**71a**)	98	[177]
	PFL	$MeCO_2CH{=}CH_2$	$MeCO_2CH{=}CH_2$	2 (**71a**)	94	[178,179]
	PFL	$tBuCO_2CH{=}CH_2$/THF	$tBuCO_2CH{=}CH_2$	2 (**71b**)	73	[180]
				2 (**71c**)	76	
72  a: R=OMe b: R=SEt	PSL	$RCO_2CH_2CF_3$/THF	$RCO_2CH_2CF_3$	3 (**72a**)		[176]
		$RCO_2CH{=}CH_2$/THF	$RCO_2CH{=}CH_2$	3 (**72a**)		
	PSL	$MeCO_2CH{=}CH_2$	$MeCO_2CH{=}CH_2$	3 (**72a**)	86	[177]
	PFL	$MeCO_2CH{=}CH_2$	$MeCO_2CH{=}CH_2$	3 (**72a**)	86	[178,179]
	PFL	$MeCO_2CH{=}CH_2$	$MeCO_2CH{=}CH_2$	3 (**72b**)	86	[180,181]
73 	PPL	THF/pyridine (4:1)	$PrCO_2CH_2CF_3$	2	93	[182]
74  a: R=butyryl b: R=trityl c: R=benzyl	PPL	THF/pyridine (4:1)	$PrCO_2CH_2CF_3$	2 (**74a**)	84	[165,182]
	PFL	THF/pyridine (4:1)	$PrCO_2CH_2CF_3$	2 (**74a**)	81	[165]
	CCL	CH_2Cl_2/pyridine (4:1)	$PrCO_2CH_2CF_3$	2 (**74a**)	80	[165]
	PFL	$MeCO_2CH{=}CH_2$	$MeCO_2CH{=}CH_2$	2 (**74b**)		[175]
	PFL	$MeCO_2CH{=}CH_2$	$MeCO_2CH{=}CH_2$	2 (**74c**)		[175]
75 	PFL	$MeCO_2CH{=}CH_2$	$MeCO_2CH{=}CH_2$	3		[175]

Table 18-2. (cont.).

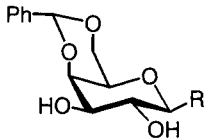
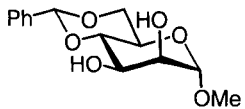
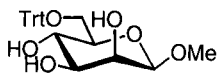
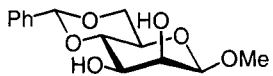
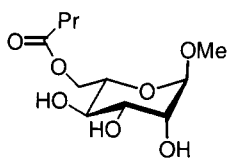
Compound No.	Structure	Enzyme[a]	Solvent	Acyl Donor	Position	Yield (%)	Ref.
76	 a: R=OAll b: R=SEt	PSL	$MeCO_2CH{=}CH_2$	$MeCO_2CH{=}CH_2$	3 (**76a**)	91	[177]
		PFL	$MeCO_2CH{=}CH_2$/THF	$MeCO_2CH{=}CH_2$	3 (**76b**)	10	[180]
77		PSL	$MeCO_2CH{=}CH_2$	$MeCO_2CH{=}CH_2$	2	90	[177]
78		PFL	$MeCO_2CH{=}CH_2$	$MeCO_2CH{=}CH_2$	3		[175]
79		PSL	$RCO_2CH{=}CH_2$	$RCO_2CH{=}CH_2$	3	92	[177]
80		PPL	THF	$PrCO_2CH_2CF_3$	4	65	[165]
		PFL	THF	$PrCO_2CH_2CF_3$	4	68	[165]

Table 18-2. (cont.).

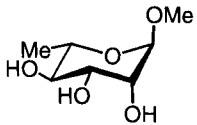
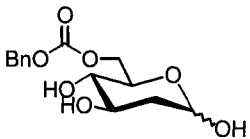
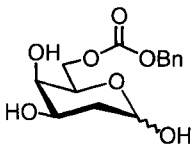
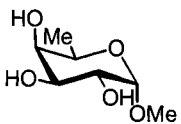
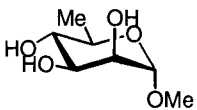
Compound No.	Structure	Enzyme[a]	Solvent	Acyl Donor	Position	Yield (%)	Ref.
81		PPL	THF/$PrCO_2CH_2CF_3$ (4:1)	$PrCO_2CH_2CF_3$	4	70	[183]
82		PSL	dioxane	$RCO_2N{=}CMe_2$	3	54–67	[156]
84		PSL	dioxane	$RCO_2N{=}CMe_2$	3	48–56	[156]
84		PPL PFL	THF/pyridine (4:1) THF/pyridine (4:1)	$PrCO_2CH_2CF_3$ $PrCO_2CH_2CF_3$	2 2	78 84	[184] [184]
85		PFL	THF	$PrCO_2CH_2CF_3$	2	40	[184]

Table 18-2. (cont.).

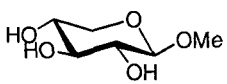
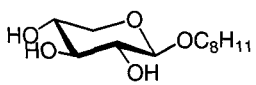
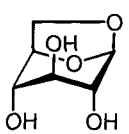
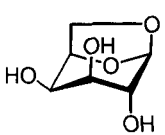
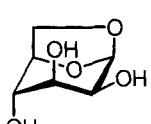
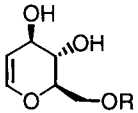
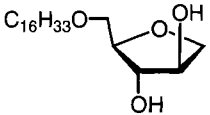
Compound No.	Structure	Enzyme[a]	Solvent	Acyl Donor	Position	Yield (%)	Ref.
86		PSL	MeCN	$MeCO_2CH{=}CH_2$	3,4	85	[185]
87		PSL	Hexane	$MeCO_2CH{=}CH_2$	2,4 3,4	70 28	[185]
88		CAL CAL PSL CAL	dioxane dioxane $MeCO_2CH{=}CH_2$ $PrCO_2Et/tBuOH$	$RCO_2N{=}CMe_2$ $MeOCO_2N{=}CMe_2$ $MeCO_2CH{=}CH_2$ $PrCO_2Et$	4 4 4 4; diester	70–72 42	[156] [156] [186,187] [188]
89		CAL	$PrCO_2Et$	$PrCO_2Et$	4		[188]
90		PSL	$MeCO_2CH{=}CH_2$	$MeCO_2CH{=}CH_2$	4		[187,189]

Table 18-2. (cont.).

Compound No.	Structure	Enzyme[a]	Solvent	Acyl Donor	Position	Yield (%)	Ref.
91	 **a**: R=acetyl **b**: R=benzoyl **c**: R=PhAc	PFL	DME	$RCO_2CH{=}CH_2$	3	84–93	[190]
92		HLL	benzene	$PrCO_2CH_2CCl_3$	2	66	[191]
		RJL	benzene	$PrCO_2CH_2CCl_3$	3	79	[191]

a Many enzymes were normally screened for activity, only the best results are listed. ANL: *Aspergillus niger* lipase; CAL: *Candida antarctica* lipase; CCL: lipase from *Candida cylindracea* (later renamed *Candida rugosa*; CRL); CVL: *Chromobacterium viscosum* lipase; HLL: *Humicula lanuginosa* lipase; PFL: *Pseudomonas fluorescens* (later renamed *Pseudomonas cepacia*) lipase; PPL: porcine pancreas lipase; PSL: *Pseudomonas cepacia* lipase; RJL: *Rhizopus japonicus* lipase.

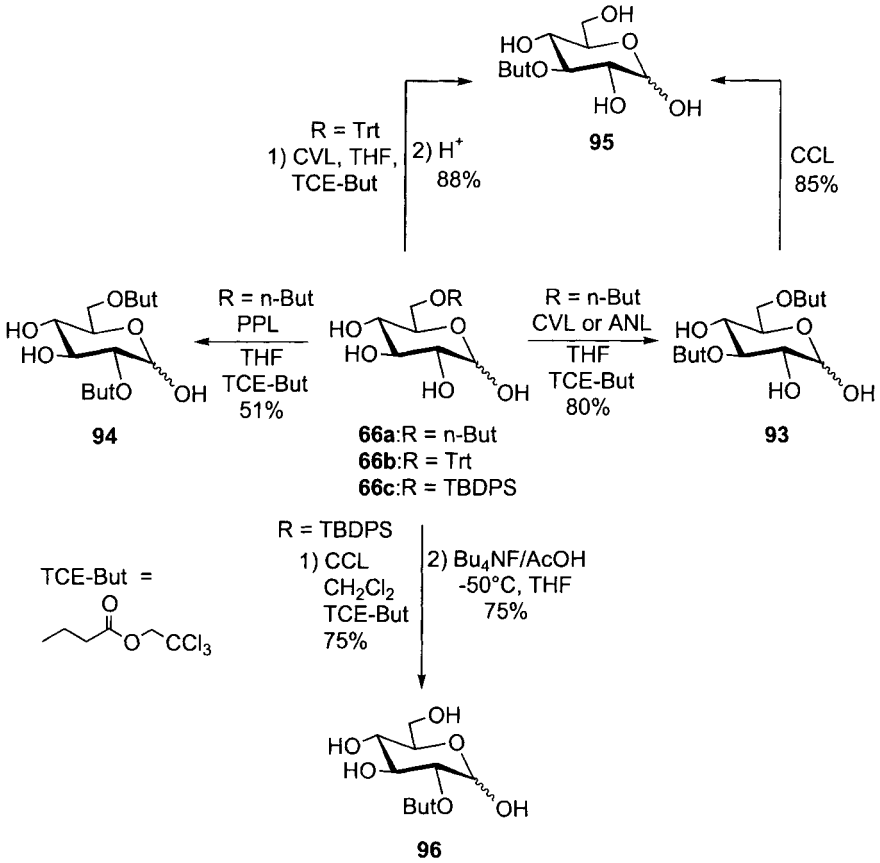


Figure 18-15. Selective enzymatic introduction of protecting groups into partially acylated hexoses.

particularly suitable for the enzymatic reactions. This was also observed in the lipase-mediated acylation of the methyl glycosides of both D- and L-fucose and -rhamnose, respectively[184]. Using lipase from *Pseudomonas fluorescence* (PFL), both D-carbohydrates were converted into the 2-monobutanoates with high regioselectivity. The naturally occurring L-enantiomers of these 6-deoxysugars, however, were esterified preferably at the 4-hydroxy groups. These results contrast favorably with chemical derivatizations, since the 4-hydroxy groups of the 6-deoxy-L-carbohydrates have only slight reactivity toward chemical acylating reagents. In addition, methyl-L-fucoside can be converted into the 3-butanoate with lipase from *Candida cylindracea*. The introduction of an acyl-substituent into the 6-positions of the D-fucoside and the L-rhamnoside does not influence the regioselectivity of the enzymatic acylation[165].

Finally, it should be mentioned, that some attempts were made to differentiate between the hydroxy groups of fructose by enzymatic methods, however, with lipases as well as with subtilisin, only mixtures of the 1- and 6-isomers were ob-

tained[141, 150, 192]. Regioselectively monosubstituted fructoses can, however, be obtained by an enzymatic approach from sucrose (*vide infra*).

18.5.2
Deprotection of Monosaccharides[133, 137]

Initial attempts to apply lipases for the enzymatic removal of acyl groups from glucose pentaacetate only resulted in low levels of selectivity[193, 194]. However, later on lipase from porcine pancreas (PPL)[168] was found to hydrolyze exclusively the anomeric acetate from peracetylated pyranoses while the esterase from *Rhodosporium toruloides* (RTE)[195] releases the primary hydroxy group in preferance (Table 18-4). On the other hand, if the anomeric center is derivatized as a methyl glycoside, the regioselective enzymatic liberation of the 6-OH group becomes feasible with a number of hydrolytic enzymes[168, 195–199]. Thus, from methyl α-D-glucose tetraoctanoate **97a** and the corresponding tetrapentanoate **97b**, lipase from *Candida cylindracea* (CCL) removes only the primary ester group in yields of ca. 75%. Similarly, the α-D-galactoside **103**, as well as the corresponding mannoside **104b** and the 2-acetamido-2-deoxy-mannoside **105** were converted into the 6-deprotected pyranosides in 29–50 % yield (Table 18-3), but the 2-acetamido-2-deoxy-glucoside was only a poor substrate. In the latter cases the regioselectivity was less pronounced and the 4,6-dideoxy derivatives were also formed in ca. 20% yield. In addition to this class of compounds, lipases also accept hexopyranosides carrying several different functionalities (e.g. acetals[197], enol ethers[169, 200] and, in particular, 1,6-anhydropyranoses as substrates (Tables 18-3 and 18-4). In all cases the reaction conditions are so mild that the acid sensitive structures of these compounds remain unaffected. Particularly remarkable is the regioselectivity displayed by lipase from *Pseudomonas cepacia* (PSL) in the deprotection of the glycal **131**[169, 200]. The biocatalyst exclusively attacks the 3-acetate and leaves the primary ester intact. The enzymatic deprotection strategy can also be used to synthesize carbohydrates carrying a single acyl group in selected positions. Thus, 3,6-dibutyryl glucose **93** (prepared by enzymatic acylation of glucose) was converted into the 3-butanoate **95** by lipase mediated hydrolysis of the 6-ester (Fig. 8-15)[164]. The principles and the enzymes mentioned above which allow the regio- and chemoselective protection and deprotection of the various pyranoses to be carried out were also successfully applied to the enzymatic manipulation of acyl groups in furanoses. Of particular interest in this context is the finding that the five-membered rings can also be handled by the biocatalysts with a pronounced regioselectivity, although furanoses can adopt more flexible conformations with similar energies in solution.

The cleavage of the primary acetyl groups from the furanosides **106–111** could be carried out in high yields with lipase from *Candida cylindracea* (Table 18-3)[168]. For the 2-deoxy-α-D-ribofuranoside and the α- and the β-xylo-compounds the hydrolysis was less selective. From the peracetylated furanoses **125** and **126** the anomeric acyl group was removed with total selectivity by means of lipase from *Aspergillus niger* (Table 18-4).

1,6-Anhydropyranoses serve as convenient starting materials for various synthetic

Table 18-3. Selective deacylation of primary hydroxy groups in monosaccharides.

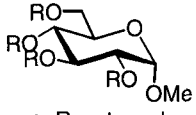
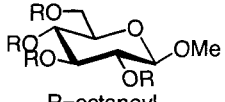
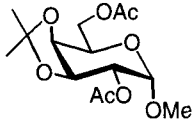
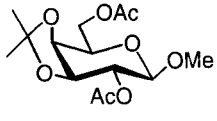
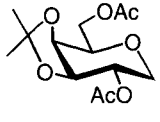
Compound No.	Structure	Enzyme[a]	Solvent	Position	Yield (%)	Ref.
97	 **a**: R=octanoyl **b**: R=pentanoyl **c**: R=acetyl	CCL	0.1 M phosphate buffer	6	78 (**97a**)	[168]
		CCL	0.1 M phosphate buffer	6	75 (**97b**)	[168]
		CCL	0.1 M phosphate buffer	6	90 (**97b**)	[196]
		CCL	0.1 M phosphate buffer, Bu_2O (10%)	6	(**97c**)	[197]
		PEG-modified CCL	Cl_3CCH_3	6	27 (**97c**)	[198]
				4,6	48 (**97c**)	
		CRL	0.1 M phosphate buffer	6	91 (**97c**)	[199]
		RTE	citrate buffer	6	77 (**97c**)	[195]
98	 R=octanoyl	CCL	0.1 M phosphate buffer	6	77	[168]
99		PPL	0.1 M phosphate buffer, acetone 10:1	6	90	[166]
100		PPL	0.1 M phosphate buffer, acetone 10:1	6	82	[166]
101		PPL	0.1 M phosphate buffer, acetone 10:1	6	75	[166]

Table 18-3. (cont.).

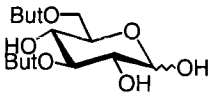
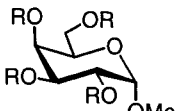
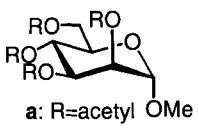
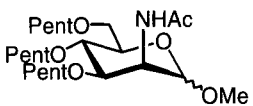
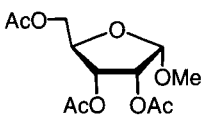
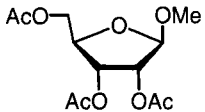
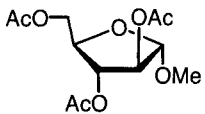
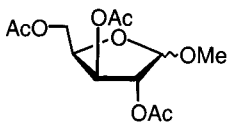
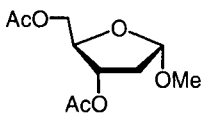
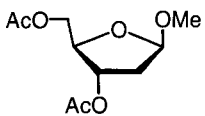
Compound No.	Structure	Enzyme[a]	Solvent	Position	Yield (%)	Ref.
102		CCL	0.1 M Tris·HCl	6	85	[164]
103	 a: R=acetyl b: R=pentanoyl	RTE	citrate buffer	6	85 (**103a**)	[195]
		CCL	0.1 M phosphate buffer	6	29 (**103b**)	[168]
104	 a: R=acetyl b: R=pentanoyl	CRL	0.1 M phosphate buffer	6	94 (**104a**)	[199]
		RTE	citrate buffer	6	70 (**104a**)	[195]
		CCL	0.1 M phosphate buffer	6	33 (**104b**)	[168]
105		CCL	0.1 M phosphate buffer	6	50	[168]
106		CCL	0.1 M phosphate buffer, 10% DMF	6	85	[168]

Table 18-3. (cont.).

Compound No.	Structure	Enzyme[a]	Solvent	Position	Yield (%)	Ref.
107		CCL	0.1 M phosphate buffer, 10% DMF	5	96	[168]
108		CCL	0.1 M phosphate buffer, 10% DMF	5	98	[168]
109		CCL	0.1 M phosphate buffer, 10% DMF	5 3	50 30	[168]
110		CCL	0.1 M phosphate buffer, 10% DMF	5 3	40 50	[168]
111		CCL	0.1 M phosphate buffer, 10% DMF	5	63	[168]

a Many enzymes were normally screened for activity, only the best results are listed. ANL: *Aspergillus niger* lipase; CCL: lipase from *Candida cylindracea* (later renamed *Candida rugosa*; CRL); PPL: porcine pancreas lipase; RTE: *Rhodosporium toruloides* esterase.

Table 18-4. Selective deacylation of secondary hydroxy groups in monosaccharides.

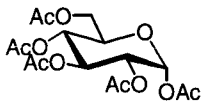
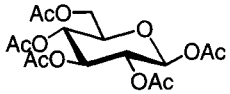
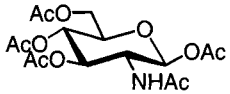
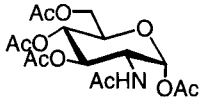
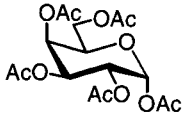
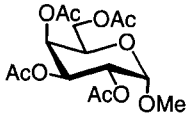
Compound No.	Structure	Enzyme[a]	Solvent	Position	Yield (%)	Ref.
112		CCL	0.1 M phosphate buffer	4,6	73	[168]
		RTE	citrate buffer	6	54	[195]
		PPL	0.05 M phosphate buffer, 10% DMF	1	70	[168]
		PFL	phosphate buffer, MeCN (7:3)	1	80	[201]
		CCL	phosphate buffer, MeCN (7:3)	4	50	[201]
		CCL	phosphate buffer, MeCN (7:3)	6	75	[201]
113		PPL	0.05 M phosphate buffer, 10% DMF	1		[168]
		CAL	butanone	1	95	[202]
114		PPL	0.05 M phosphate buffer, 10% DMF	1	96	[168]
115		ANL	phosphate buffer, MeCN (10:1)	1,4	41	[203]
		RTE	citrate buffer	6	80	[204]
116		PPL	0.05 M phosphate buffer, 10% DMF	1	75	[168]
		RTE	citrate buffer	6	67	[195]
117		ANL	0.1 M phosphate buffer, 10% acetone	2	58	[205]

Table 18-4. (cont.).

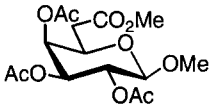
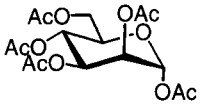
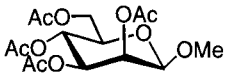
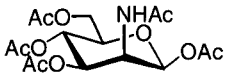
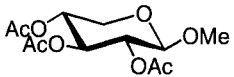
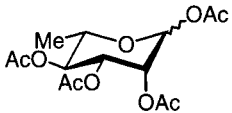
Compound No.	Structure	Enzyme[a]	Solvent	Position	Yield (%)	Ref.
118		*hog kidney* acylase	phosphate buffer, DMF (10:1)	2	93	[206]
		Aspergillus niger pectinase	phosphate buffer, DMF (10:1)	3	27	[206]
		ANL	phosphate buffer, DMF (10:1)	4	11	[206]
119		PPL	0.05 M phosphate buffer, 10% DMF	1	95	[168]
		RTE	citrate buffer	6	88	[195]
120		ANL	0.1 M phosphate buffer, 10% acetone	3	61	[205]
121		PPL	0.05 M phosphate buffer, 10% DMF	1	88	[168]
122		PSL	*t*Amyl-OH	4	84	[207]
		PEG-modified CCL	Cl_3CCH_3	4	82	[198]
123		PPL	0.05 M phosphate buffer, 10% DMF	1	54	[168]

Table 18-4. (cont.).

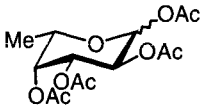
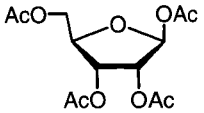
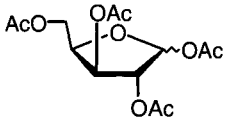
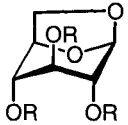
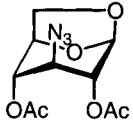
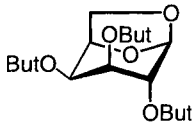
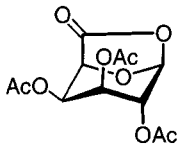
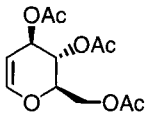
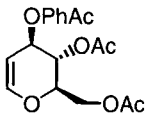
Compound No.	Structure	Enzyme[a]	Solvent	Position	Yield (%)	Ref.
124		PPL	0.05 M phosphate buffer, 10% DMF	1	71	[168]
125		ANL	0.1 M phosphate buffer, 10% DMF	1	63	[168]
126		ANL	0.1 M phosphate buffer, 10% DMF	1	50	[168]
127	 a: R=acetyl b: R=butyryl	RJL	0.1 M phosphate buffer	2	47 (127a)	[208]
				4	15 (127a)	
		WGL	0.1 M phosphate buffer	3	67 (127a)	[208]
		PLE	0.1 M phosphate buffer	4	69 (127a)	[208]
		PPL	0.05 M citrate-phosphate buffer	4	42 (127a)	[209]
		CVL	0.1 M phosphate buffer	4	91 (127b)	[210]
		CCL	0.1 M phosphate buffer	2,4	77 (127b)	[210]
128		CCL	0.1 M phosphate buffer	4	85–90	[211]
		alcalase	0.1 M phosphate buffer	2	82	[211]

Table 18-4. (cont.).

Compound No.	Structure	Enzyme[a]	Solvent	Position	Yield (%)	Ref.
129		CCL	0.1 M phosphate buffer	2		[210]
		CCL	0.1 M phosphate buffer	2	90	[212]
		PPL	0.1 M phosphate buffer	2	16	[212]
				4	19	
				2,4	65	
130		WGL	phosphate buffer, DMF (10:1)	2	60	[206]
131		PSL	0.25 M phosphate buffer	3	90	[169]
		acetyl esterase from the flavedo of oranges	0.15 M NaCl buffer	3,4	24	[74,213]
				3,4,6	22	
132		PGA	0.1 M phosphate buffer	3	80–85	[190]

a Many enzymes were normally screened for activity, only the best results are listed. ANL: *Aspergillus niger* lipase; CAL: *Candida antarctica* lipase; CCL: lipase from *Candida cylindracea* (later renamed *Candida rugosa*; CRL); PGA: penicillin-G-acylase; PLE: porcine liver esterase; PPL: porcine pancreas lipase; PSL: *Pseudomonas cepacia* lipase; RJL: *Rhizopus japonicus* lipase; RTE: *Rhodosporium toruloides* esterase; WGL: wheat germ lipase.

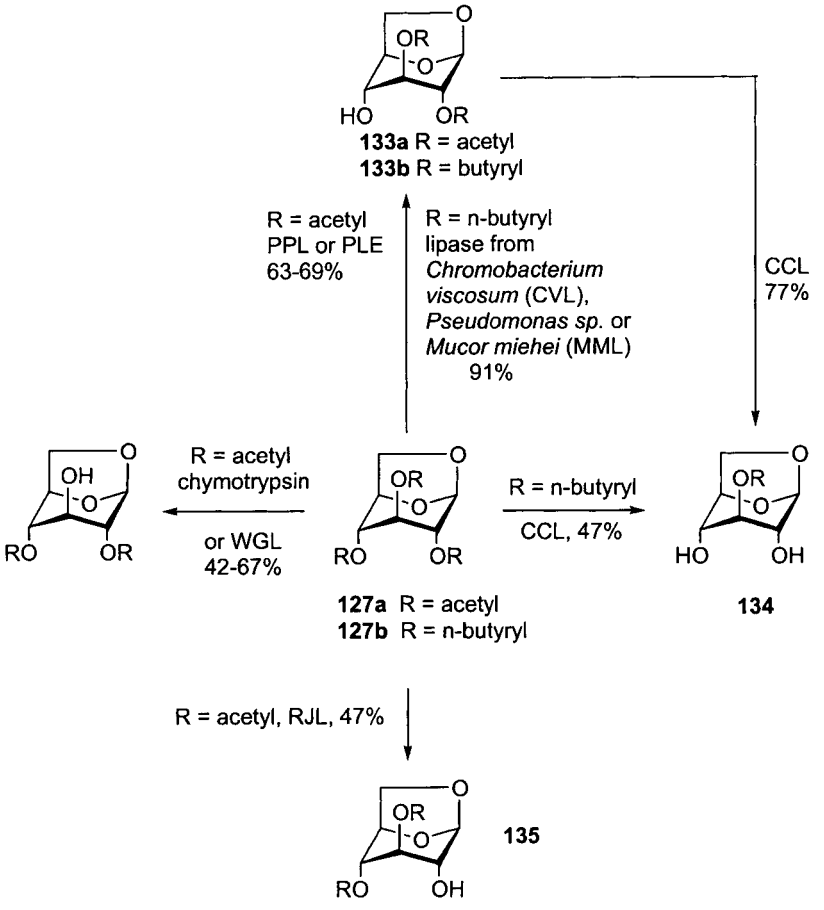


Figure 18-16. Selective enzymatic removal of protecting groups from 1,6-anhydropyranoses.

purposes in carbohydrate chemistry. Therefore, the directed manipulation of their hydroxy groups is of particular interest. Each of the three OH-groups in 1,6-anhydroglucopyranose can be liberated selectively making use of enzymatic reactions (Fig. 18-16, Table 18-4)[208–210, 212]. Thus, the 4-protecting group was split off from the triacetate **127a** using lipase from porcine pancreas (PPL)[209] or pig liver esterase (PLE)[208, 209]. The acetate in the 3-position could be attacked preferentially using chymotrypsin[209] or lipase from wheat germ (WGL)[208], and the 3,4-diacetate **135** was obtained by hydrolysis with lipase from *Rhizopus javanicus* (RJL)[208]. In each case, however, other derivatives were formed as undesired by products. High yields could be obtained from the tri-n-butanoate **127b**. It was converted into the 2,3-dibutanoate **133b** in 91% yield by means of several lipases, but the enzyme from *Candida cylindracea* (CCL) removed two acyl groups successively to yield the monobutanoate **134**. Similarly, the analogous 3-azido-1,6-anhydropyranose **128** is regioselectively deacylated at O2 and O4 by means of lipase OF from *Candida cylindracea* and

alcalase, respectively[211]. Of particular importance is the stereochemistry at C4 of the bicyclic substrates. If the alcohol at this position is equatorial, as for instance in the corresponding 1,6-anhydrogalactopyranose **129** and the analogous lactone **130**, several enzymes act only in a random fashion or not at all[210]. However, the acyl group in the 2-position seems to be preferred (Table 18-4). The results obtained from these studies indicate that the reactivity of acyl protecting groups in 1,6-anhydropyranoses toward hydrolysis by lipases decreases in the order $C4_{ax} > C2_{ax} > C3_{ax} >> C4_{eq}$.

The above mentioned investigations revealed that the lipase-mediated hydrolysis proceeds at higher reaction rate and, in many cases with better selectivity, if butanoates or pentanoates are employed as substrates instead of acetates. However, the use of enzymatic deacylations is by no means restricted to simple alkanoates. An illustrative and impressive example is found in the hydrolysis of generally base-stable carbohydrate pivaloylates using an esterase from rabbit serum (ERS)[214–217]. For instance, the biocatalyst selectively splits off the 6-pivaloyl group from α-methyl 3,4,6-tripivaloyl-2-acetamido-2-deoxy-glucoside. On prolonged incubation the complete removal of pivaloylates from carbohydrates is also possible. Of particular significance is, that the enzyme does not have to be purified, but that crude serum preparations are sufficient for the preparative purposes. A further enzyme which allows the chemo- and regioselective unmasking of different carbohydrate derivatives to be carried out is acetyl esterase from the flavedo of oranges, a biocatalyst which preferably hydrolyzes acetic acid esters[32, 218]. It can be applied for the synthesis of selectively deacylated pyranoses. Thus, from pentaacetylglucose **112** the 2,3,4,6-tetraacetate is obtained by means of the regioselective saponification of the 1-acetate. If the hydrolysis is allowed to proceed further, the 6-acetate is also cleaved and the 2,3,4-triacetate becomes available in ca. 40% yield. If tri-*O*-acetyl-glucal **131** is subjected to the enzymatic hydrolysis, at 40% conversion the 6-acetate is the main product.

By introducing acyl groups which are specifically recognized by certain enzymes into carbohydrates, not only the regioselectivity but also the chemoselectivity of the biocatalysts can be exploited. This can, for instance, be achieved by the selective saponification of phenylacetates catalyzed by penicillin G acylase[30–32]. The enzyme liberates the 2-OH group of 1,3,4,6-tetraacetyl-2-phenylacetyl glucose without affecting the acetic acid esters. In this case, moreover, an ester of a secondary hydroxy function is chemoselectively hydrolyzed in the presence of the chemically more reactive acetates at the 6-position and at the anomeric center. This approach was also adopted for the enzymatic deprotection of the glucal **132**. Thus, its 3-OH group was liberated without cleaving the acetates that were present[190].

18.5.3
Di- and Oligosaccharides[137]

For enzymatic protecting group manipulations on di- and oligosaccharides in particular the use of subtilisin together with dimethylformamide as the solvent is advantageous. As has already been pointed out, the use of DMF is often critical, since

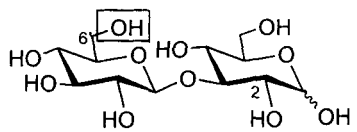
its dissolving ability is high enough to solubilize even highly polar polyhydroxy compounds (comparable experiments with pyridine as the solvent generally failed)[141]. Only a few reports about the successful use of other solvents such as pyridine[219] or *tert*-butanol[220] have been published.

Subtilisin accepts several disaccharides as substrates and transfers butyric acid from ethyl or trichloroethyl butanoate to the primary 6'-hydroxy functions of the nonreducing monosaccharide of the β-(1–3)-linked cellobiose **136** and the respective maltobiose (Fig. 18-17)[150, 220]. For lactose the regioselectivity was less pronounced, however, methyl and benzyl β-D-lactoside **137** were converted into the 6'-butanoates in 71–73% yield[221]. Rutinose in which the primary hydroxy group of the glucose moiety is blocked (see also **149**, Fig. 18-19), is selectively substituted in the 3-position[222]. In addition, higher maltooligomers could also be acylated in the 6-position of the terminal nonreducing carbohydrate. For instance, 6''-*O*-butyrylmaltotriose was isolated in 29% yield, but also the corresponding tetra-, penta-, hexa- and heptamer were substrates for the biocatalyst. These enzymatic esterifications open a route to discriminating between the primary hydroxy groups in di- and oligosaccharides in a convenient and straightforward way. Classical chemical one step methods of comparable selectivity are not available for this purpose[139, 140], and multistep sequences usually have to be carried out if the selective protection of a specific primary hydroxy group in a di- or oligosaccharide is desired.

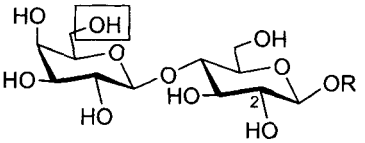
Owing to its great commercial importance as a renewable resource, sucrose **138** has been subjected to several enzymatic hydroxy group manipulations. This nonreducing disaccharide turned out to be a substrate for subtilisin also[150]. In contrast to chemical acylations in which the most reactive OH-groups are found in the 6- and the 6'-position, the enzyme selectively transfers various acyl functions to the 1'-alcohol (Fig. 18-17)[150, 192, 223]. This acylation was usually carried out in DMF as a solvent, but the use of anhydrous pyridine gave similar results[219]. The monoacylated disaccharides **139** thereby obtained could then be further transformed enzymatically. On the one hand, with the lipase from *Chromobacterium viscosum* (CVL) the free primary 6-OH group was acylated in 31% yield. On the other hand, the 1'-esters **139** are substrates for yeast α-glucosidase which hydrolyzes the glycosidic bond and thus makes the 1-*O*-acylfructoses **140**, potentially useful as chiral synthons, available[192]. Alternatively, the 6'-OH-group in sucrose **138** can be selectively acylated, if the carbohydrate is converted into the 2,1':4,6-bisacetal prior to the treatment with a lipase (Novozym™ 435)[224].

On considering hydrolysis, several enzymes were investigated[225–229]. Depending on the biocatalyst used, acetyl groups from different positions of octaacetyl sucrose **141** could be removed selectively in useful yields. For instance, alcalase and protease N preferably attack the acetate on O1'[226, 230], the lipase from *Candida cylindracea* preferably liberates the OH-group on C4' of the furanoid ring[225, 230] and wheat germ lipase preferentially liberates the 1'-, 4'- and 6'-OH-groups (Fig. 18-17)[223, 231].

The deacylation of the octaacetates of cellobiose, lactose, maltose and melibiose with *Aspergillus niger* lipase leads to the formation of the respective carbohydrate heptaacetates with a free anomeric OH-group at C1 in high yield[230, 232]. With



136 cellobiose 47%



137 lactosides 71-73% R = Me, Bzl

subtilisin, trichloroethyl butyrate, DMF

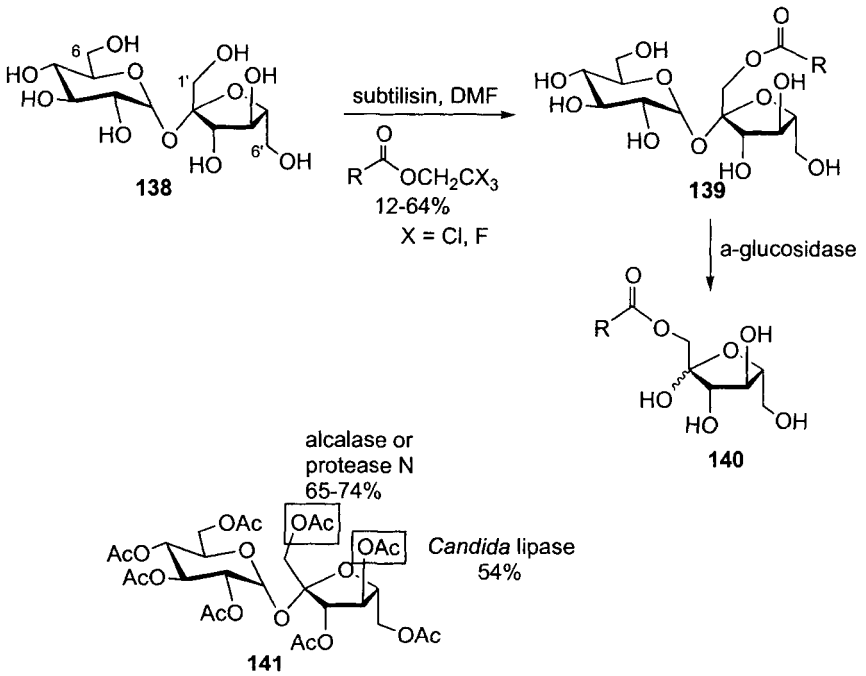


Figure 18-17. Selective enzymatic protection and deprotection of disaccharides.

prolonged reaction times, the acetates at C1 and C2 are hydrolyzed from cellobiose and lactose octaacetate in 51% or 42% yield, respectively.

18.5.4
Nucleosides[135, 233]

The directed protection of nucleoside functional groups is a fundamental problem in nucleoside and nucleotide chemistry. Although several chemical methods are available for the regioselective acylation of the nucleoside carbohydrates, enzymatic

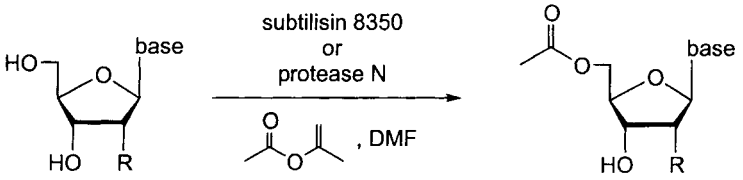
methods offer significant advantages with respect to yield, regioselectivity and the number of synthetic steps which have to be carried out.

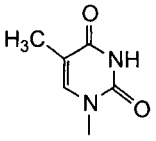
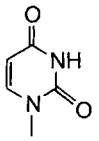
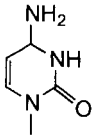
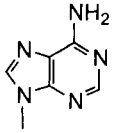
Earlier studies focussed on the use of the dihydrocinnamoyl group as an enzyme-labile nucleoside protecting function which can be removed through the agency of α-chymotrypsin[234, 235]. Although the enzyme shows an interesting tendency to attack preferably the 5'-position, this technique was not exploited further. Highly regiodiscriminating biocatalyzed acyl transfer reactions to the carbohydrate parts of various nucleosides could be carried out again employing the protease subtilisin together with dimethylformamide as solvent. In particular, a mutant of this enzyme, obtained via site specific mutations appears to display advantageous properties. It transfers the acetyl group from isopropenyl acetate to the primary hydroxy functions of various purine and pyrimidine nucleosides and 2'-deoxynucleosides **142** in high yields (Fig. 18-18)[236]. Commercially available subtilisin (protease N from Amano) provided the same compounds with identical yields and selectivities, however, five times more enzyme is required for this purpose. In addition, in the transfer of butyric acid from trichloroethyl butanoate to adenosine and uridine, carried out earlier[150], this biocatalyst showed inferior properties with respect to regioselectivity and yields.

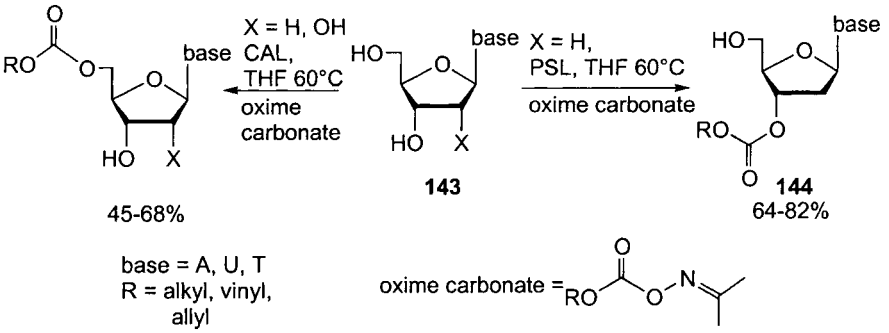
The selective introduction of protecting groups into the hydroxy functions of different nucleosides can also be achieved by means of lipases. Thus, unprotected pyrimidine and purine 2'-deoxynucleosides **143** (X = H) are selectively converted into the 3'-*O*-acylated derivatives **144** in 64–82% yield making use of lipase from *Pseudomonas cepacia* (PSL) and employing oxime carbonates as acyl donors (Fig. 18-18)[237–239]. Similarly, by applying oxime esters or acid anhydrides, different ester functions can be selectively introduced into the 3'-position of nucleotides by using the lipases from *Candida cylindracea* (CCL), porcine pancreas (PPL) or *Pseudomonas cepacia* (PSL)[240–244]. If lipase from *Candida antarctica* (CAL) is used, however, the esters and carbonates are predominantly generated at the primary 5'-OH group of (deoxy)nucleotides[238, 239, 241, 242, 244–247]. Furthermore, in the case of ribonucleotides, complete regioselectivity can be achieved by using the same methodology[241]. The regioselectivity of the CAL-catalyzed alkoxycarbonylation is profoundly influenced significantly by the structure of the starting oxime carbonate[248]. In the alkoxycarbonylation of thymidine the use of the phenyl derivative leads to almost exclusive formation of the 5' carbonate, while the corresponding allyl carbonate is introduced without any regioselectivity.

An investigation of the enzyme-catalyzed acylation of α-, xylo-, anhydro-, and arabino-nucleosides showed that in these cases the primary 5'-hydroxy group can be selectively acylated using lipase from *Candida antarctica* (CAL)[249–251]. A selective derivatization of the 3'-OH-group, however, was unsuccessful.

When acylations of nucleosides with acid anhydrides in the presence of lipase from *Pseudomonas fluorescence* (PFL) in DMF or DMSO as the solvent first proceeded, the regioselectivity was unsatisfactory[252]. However, this lipase together with subtilisin can be utilized to effect highly specific deacylations of various pyrimidine nucleosides **145** (Fig. 18-18)[253]. Thus, lipase from *Pseudomonas fluorescence* (PFL) preferably attacks the hexanoyl group on the secondary hydroxy function of the *N*-



R	H	OH	H OH	H OH
base				
yield [%]	quant.	90	80 80	80 65



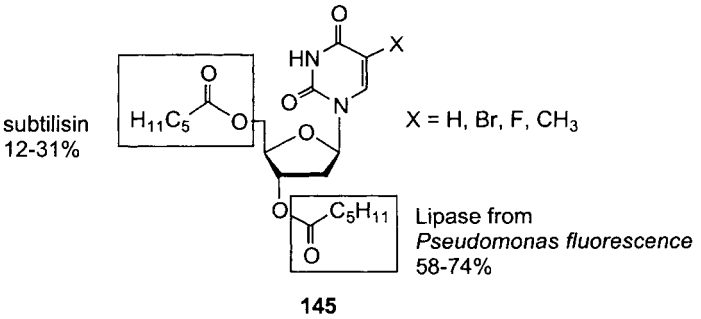


Figure 18-18. Selective enzymatic protection and deprotection of the carbohydrate parts of nucleosides.

glycosides, giving rise to the 5-esters in good yields. On the other hand, subtilisin gives rise to the 3-esters with moderate results. It should be noted, however, that in both cases from considerable to large amounts (6–71 %) of the completely deprotected nucleosides were also formed. Subtilisin in phosphate buffer also selectively hydrolyzes the 5'-acetate of purine and pyrimidine triacetylated esters to give the corresponding 2',3'-diacetylribonucleosides in 40–92 % yield[254]. A similar preference was observed for the lipase from porcine pancreas, but with poorer selectivity and a slower reaction rate. This enzyme, however, deacetylated the deoxynucleoside 3',5'-di-*O*-acetylthymidine at the 5'-position in almost quantitative yield[255]. In contrast, if lipase from *Candida cylindracea* (CCL) was used in the catalysis, the 3'-ester of this diacetate was preferentially hydrolyzed[255].

Using acetyl esterase of the flavedo of oranges, bisacylated purine deoxynucleotides can be selectively deprotected at the 3'-hydroxy group in 31–40 % yield[74]. Interestingly, by introducing a phenylacetyl group for amino protection in the purine moiety the regioselectivity of the acetyl removal is reversed. Now the primary acetate is hydrolyzed by acetyl esterase in 22–52 % yield.

In addition, the complete hydrolysis of an anomeric mixture of peracetylated 2'-deoxynucleosides by wheat germ lipase or porcine liver esterase has been used to synthesize the pure β-anomer of e.g. thymidine, this being the only completely deprotected product[256]. The alcoholysis peractylated uridines catalyzed by *Candida antarctica* lipase leads to the formation of the completely deprotected nucleotide[257]. Although this reaction can be stopped after removal of the first acetyl group, no regioselectivity was observed for the formation of di-*O*-acetyluridine.

18.5.5
Further Aglycon Glycosides

In addition to nucleosides, several other naturally occurring carbohydrate derivatives can be selectively protected/deprotected by means of enzymatic techniques. For instance, salicin **146**, a wood component that contains a primary hydroxy group located in a glucose moiety and a second one in a benzylic position, was butyrylated exclusively at the 6-OH of the monosaccharide in 35 % yield by applying subtilisin and trichloroethyl butanoate in DMF (Fig. 18-19)[150]. Under the same conditions, in riboflavin (vitamin B_2) **147** only the primary alcohol was esterified in 25 % yield[150], and colchicoside **148a** as well as a thio analog **148b** were converted into the 6'-butanoates by treatment with trichloroethyl butanoate in pyridine in the presence of subtilisin[258]. The corresponding 6'-acetates of **148a,b** were obtained by treatment with vinyl acetate in the presence of *Candida antarctica* lipase as the biocatalyst (Fig. 18-19)[162]. Similarly, the carbohydrate parts of flavonoid disaccharides were regioselectively functionalized. Thus, for instance in the disaccharide rutin **149** and the related hesperidin only the 3''-OH group of the glucose moiety was esterified upon treatment with trifluoroethyl butanoate and subtilisin in 53 % yield (Fig. 18-19)[222]. In the presence of lipase from *Candida antarctica*, however, both the 3''- and the 4'''-positions were acetylated[162]. If only the glucose moiety is present in the molecule, as in the related isoquercitrin **150**, the regioselectivty in the subtilisin-

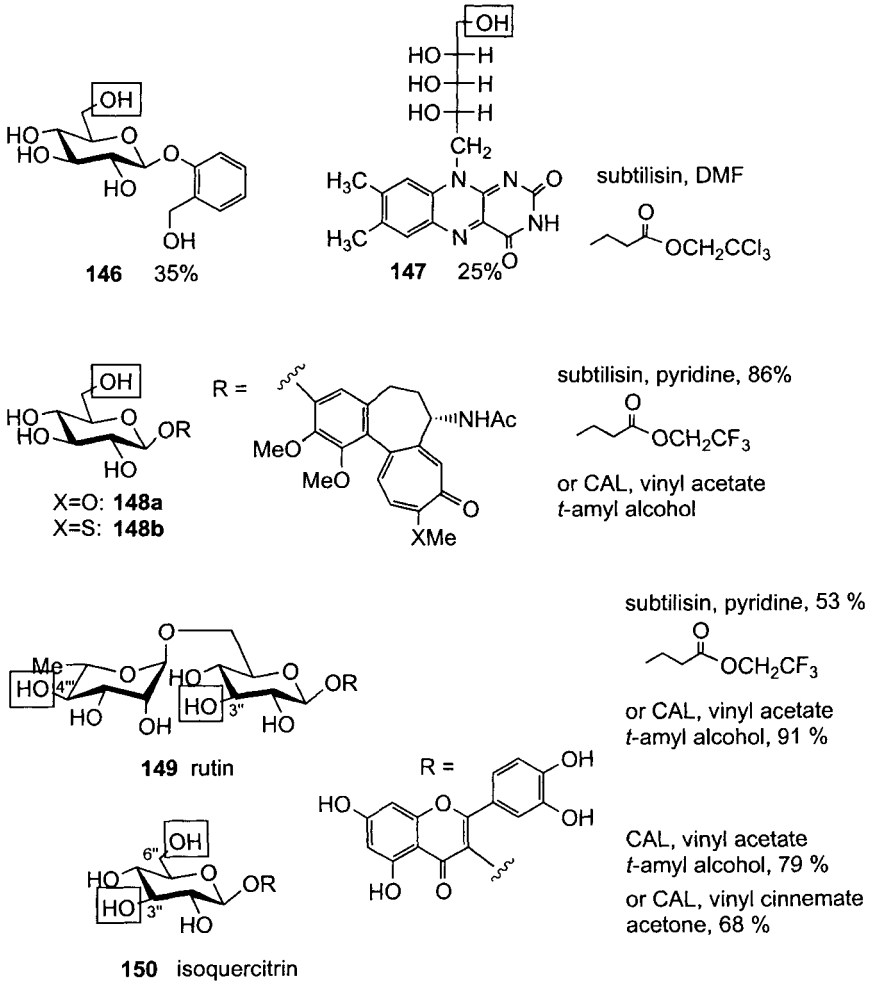
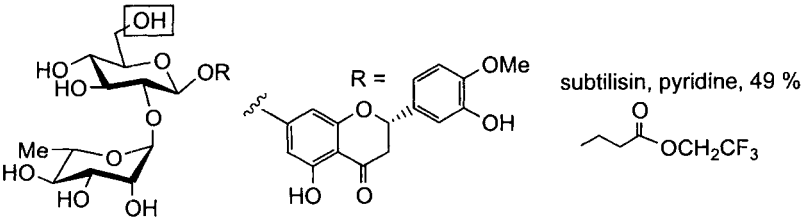


Figure 18-19. Selective enzymatic acylation of aglycon glycosides.

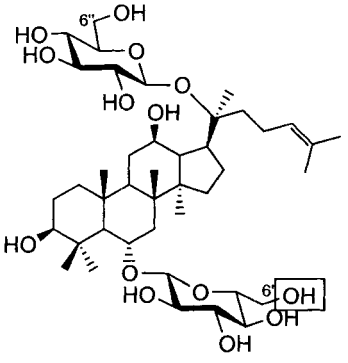
catalyzed reaction was less pronounced[259]. However, in the presence of lipase from *Candida antarctica* the 3",6"-bisacylated product is formed if vinyl acetate is used as the acyl donor[162]. Interestingly, by using vinyl cinnamate as the acyl donor, this biocatalyst only acylates the primary 6"-hydroxy group[260]. Naringine **151** was converted into the 6-glucosyl ester in the presence of subtilisin (Fig. 18-20). In all cases the rhamnose and the phenolic hydroxyls remained unattacked (for the protection of phenolic hydroxy groups in flavonoids see Sect. 18.5.8).

The steroidal glucoside ginsensoside Rg_1 **152** can be selectively monoacylated in high yields at the 6'-position using *Candida antarctica* lipase as the biocatalyst[261, 262]. In this case, similar results were obtained with different acyl donors such as vinyl acetate, dibenzyl malonate and bis(trichloethyl) malonate (Fig. 18-20).

Two impressive examples of selective enzymatic deacylations of complex sub-



151 naringin



CAL, *t*-amyl alcohol

vinyl acetate (87 %)
dibenzyl malonate (85 %)
bis(2,2,2-trichloroethyl) malonate (71 %)

152 ginsenoside Rg_1

Figure 18-20. Selective enzymatic acylation of aglycon glycosides.

strates consist in the removal of all acetates from the peracetylated ß-D-glucopyranosyl ester **153** of abscisinic acid [263] and of the gibberellinic acid derivative **154** [264], containing one glucose tetraacetate glycosidically bound and a second one attached as an ester (Fig. 18-21). In both cases the removal of the acetyl groups by chemical methods in particular was complicated by an undesired cleavage of the ester linkages to the glucoses. However, the four acetyl groups present in **153** could be hydrolyzed chemoselectively by means of helicase, an enzyme occurring in the seeds of *Helianthus annus*, whereby the unprotected glucose ester was formed in 82% yield without destroying the ester bond between abscisinic acid and glucose. Similarly, the biocatalyst removed all acetates from **154**. In this case the yield reached only 8%, it should, however, be kept in mind that ten acetic acid esters had to be cleaved in the enzymatic process and that the aglycon is rather complex.

In conclusion, the various enzyme-mediated protecting group manipulations carried out on numerous carbohydrate derivatives indicate that biocatalysts can be used advantageously in the protecting group chemistry of carbohydrates. In particular, subtilisin and several lipases from different sources (from porcine pancreas, from *Candida cylindracea, Aspergillus niger, Chromobacterium viscosum, Mucor javanicus, Pseudomonas fluorescence* and from wheat germ) allow the chemo- and regioselective acylation and deprotection of various saccharides, the structures of

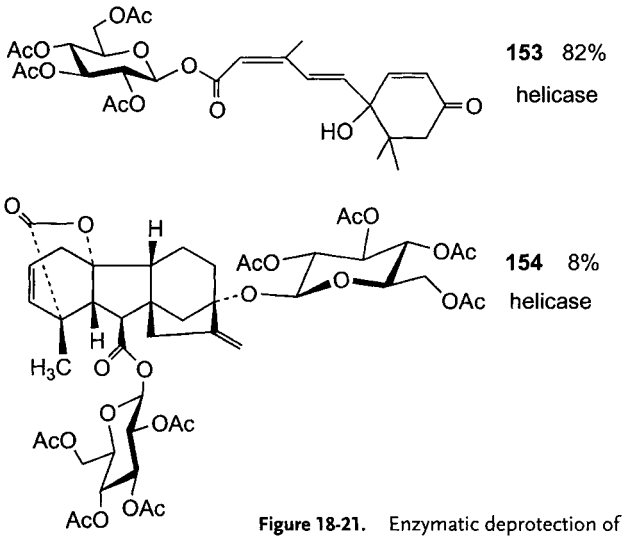


Figure 18-21. Enzymatic deprotection of complex glucosyl esters.

which differ widely, to be carried out. A general principle that emerges from these studies is that the enzymes exhibit a predominant preference toward primary hydroxy groups. If these functionalities are not present or protected, the biocatalysts are capable of selectively manipulating secondary hydroxy groups or the esters thereof. In the introduction and removal of acyl groups, the regioselectivity displayed by the enzymes often parallels the findings recorded for classical chemical transformations, although it is significantly higher in many cases. Furthermore, in several cases regioselectivities were observed in the biocatalyzed processes which can not or only slightly be achieved by means of chemical methods. Finally, it should be realized that subtilisin and the lipases are capable of introducing specific acyl groups into the carbohydrates which can later be removed selectively by different enzymatic or chemical methods.

18.5.6
Polyhydroxylated Alkaloids

The plant alkaloid castanospermine **155** and the related piperidine alkaloid 1-deoxynojirimicin **160**, like several other polyhydroxylated octahydroindolizidines, piperidines and pyrrolidines, are potent glycosidase inhibitors. These nitrogen bases are of considerable interest for the study of biosynthetic processes and, in addition, castanospermine and some of its derivatives may be of clinical value as antineoplastic agents and as drugs in the treatment of AIDS.

In the light of the analogy between the structures of these alkaloids and glucose, some of the above mentioned enzymatic methods for the selective functionalization of carbohydrates were applied to prepare several acyl derivatives of **155** and **160**. Thus, subtilisin transfers the acyl moieties from several activated esters to the 1-OH group of the bicyclic base in moderate to high yields (Fig. 18-22)[265, 266]. Again,

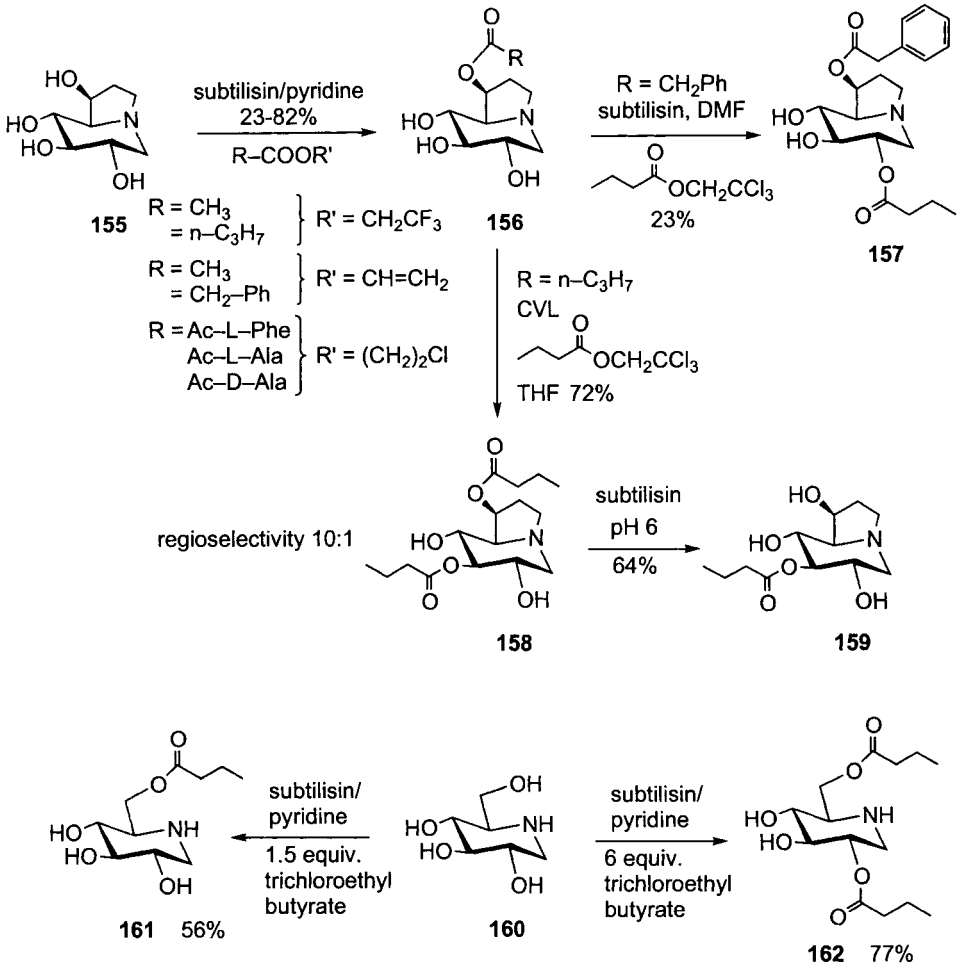


Figure 18-22. Selective enzymatic protection of polyhydroxylated alkaloids.

pyridine had to be used as the solvent for the polyhydroxy compound. The monoesters **156** obtained by this technique, like the monoesters of hexoses could subsequently be dissolved in THF and were further acylated by means of different enzymes, e. g. to the 6-butanoate **157** and the 1,7-dibutanoate **158**. Finally, the 1-ester was removed from **158** by subtilisin in aqueous solution to deliver the 7-butanoate **159** in 64% yield.

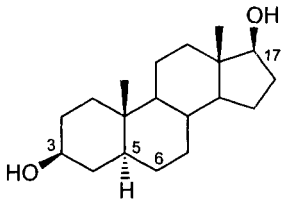
In contrast to castanospermine, 1-deoxynojirimicine **160** contains a primary hydroxy group as well as a much more nucleophilic amino function. If a small excess of trifluoroethyl butanoate is employed, subtilisin converts this alkaloid preferably into the 6-monoester **161** (Fig. 18-22)[266]. However, with 6 equiv. of the acylating agent, the 2,6-diester **162** is formed in 77% yield. This diester **162** may be subsequently deacylated regioselectively at the 6-position by means of several different enzymes.

It should be noted that under the conditions of the enzymatic acylation the amino group is not derivatized, an observation which has also been made in related cases[266, 267], e.g. *N*-terminally deprotected serine-peptides.

18.5.7
Steroids

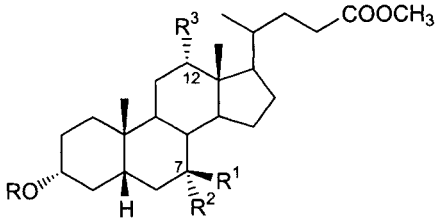
Enzymatic acyl transfer reactions are also practical processes for the acylation of hydroxy groups in steroids. The lipase from *Chromobacterium viscosum* (CVL) for instance selectively transfers butyric acid from trifluoroethyl butanoate to equatorial (ß) C3-alcoholic functions that are present in a variety of sterols, e.g. **163** and the respective 5,6-didehydro compound (Fig. 18-23)[268]. Axially oriented alcohols at C3 and secondary alcohols at C17 or in the sterol side chains are not derivatized. In addition to the equatorial alcohols, the compounds being accepted as substrates by the lipase must have the A/B-ring fusion in the *trans* configuration. In the B-ring a double bond is tolerated, in the A-ring, however, it is not. Similarly, lipase from *Candida antarctica* acylates the 3-hydroxy group in steroids such as **163** and its 5,6-didehydro derivative[269]. Interestingly, acylation in this position is preferred regardless of the orientation of the hydroxy group. For instance, treatment of **164** with vinyl acetate in the presence of *Candida antarctica* lipase leads to the formation of corresponding 3-acetylated derivative in 82% yield. In contrast, subtilisin does not recognize the hydroxy group at C3 of the steroid nucleus, but rather transfers the acyl moiety to alcoholic groups in the 17-position or in the side chains (Fig. 18-23). Changes in the A- or in the B-ring do not dramatically influence the selective mode of action of this biocatalyst. This behavior is the same as that determined for the lipase of *Pseudomonas cepacia*, which was recently used for the regio- and stereoselective acylation of steroids[270]. Thus, using these enzymes, the completely regioselective protection of either alcoholic group in several steroid diols is possible. This feature opened a route to a new chemoenzymatic process for the oxidation of selected positions of the steroid framework via an enzymatic protection/oxidation/deprotection sequence. Chemoenzymatic approaches of this type are expected to provide attractive alternatives to the currently utilized enzymatic oxidation of steroids by hydroxysteroid dehydrogenases.

A further biocatalyst comes into play when bile acids serve as starting materials, e.g. deoxycholic acid methyl ester **165**[271]. The *cis*-configuration of the A/B-ring fusion prevents the application of lipase from *Chromobacterium viscosum* (CVL) and the aliphatic chain hinders the esterification of the C12α hydroxy group by subtilisin. The lipase from *Candida cylindracea* (CCL) has proved to be the most suitable enzyme for the enzymatic acylation of bile acids. In hydrophobic solvents, i.e. hexane, toluene, butyl ether, benzene, etc. (except acetone) and employing trichloroethyl butanoate as the acyl donor, the 3α-*O*-butanoyldeoxycholic acid methyl ester **166** is formed in 80% yield without any by-products, suggesting that the enzyme is ineffective towards 12α-OH. In addition, the 7α-OH and the 7β-OH, present in **167** and **168** are not esterified by the enzyme. In both cases, the 3-butanoate is also formed (Fig. 18-23).



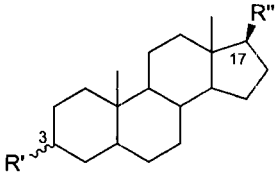
163

CVL: 3-monoburyrate 83%
CAL: 3-monoburyrate
subtilisin: 17-monobutyrate 60%

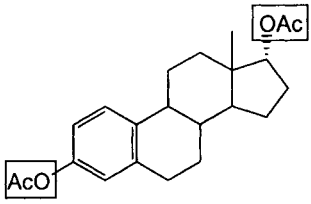


CCL, trichloroethyl butyrate, hydrophobic solvent

164 R = R^1 = R^2 = OH, R^3 = OH
165 R = R^1 = R^2 = H, R^3 = OH
166 R = But, R^1= R^2 = H, R^3 = OH 80%
167 R = But, R^1 = H, R^2 = OH, R^3 = H
168 R = But, R^1 = OH, R^2 = H, R^3 = H

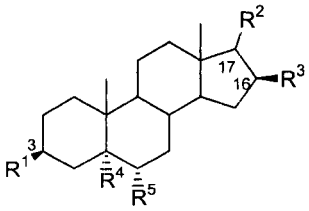


CCL:
169 R'= 3α-OAc, R"= 17β-OAc no reaction
170 R'= 3β-OAc, R"= 17β-OAc
→ 3β-OH, R" = 17β-OAc 79%

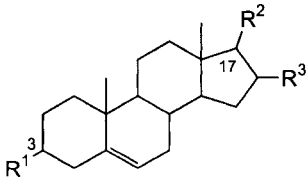


171

CCL:
3,17α-dihydroxyestradiol 60%
3-hydroxy–17α-acetoxyestradiol 25%



172 R^1=R^3=OAc, R^2=(O), R^4=R^5=H
173 R^1=R^5=OAc, R^2=(O), R^3=R^4=H
174 R^1=R^2=R^3=OAc, R^4=R^5=H



175 R^1= R^2=OAc, R^3=(O)
176 R^1=OAc, R^2 =C(O)CH_3, R^3=H

Figure 18-23. Selective enzymatic protection of steroids.

Saponification of steroid esters can also be steered with *Candida cylindracea* lipase (CCL)[272, 273]. This process occurs in the presence of octanol in organic solvents and is characterized by a pronounced stereospecificity and regioselectivity. Thus, the 3α-

esters of 3α,17β-diacetoxy steroid **169** resisted liberation, whereas the 3ß-isomer **170** is transformed into the corresponding alcohol in 79% yield. The 17α-acetate of 3,17α-diacetoxy estradiol **171** is also saponified, but at a slower rate than the 3-acetate (Fig. 18-23). In the case of the androstane derivatives **172** and **175** different selectivities of *Candida antarctica* lipase (CAL) and CCL were observed[273]. Thus, the alcoholysis of **172** in the presence of CAL afforded the C3 deprotected product in 75% yield whereas CCL led to the removal of the acetate at C16 in 66% yield. Treatment of **173**, **174** and **176** with CCL led to the cleavage of the C3 acetate in 79%, 87% and 83% yield, respectively[273, 274].

18.5.8
Phenolic Hydroxy Groups

Polyphenolic compounds occur widely distributed in nature and may possess a variety of interesting biological properties, e.g. antibiotic, antiviral and antitumor activity. The synthesis and further elaboration of these compounds often requires the selective protection or deprotection of specific phenolic hydroxy groups. To achieve this goal, the methods highlighted above for the various aliphatic polyols can also be applied successfully.

For example, for the the enzyme-catalyzed acetylation of phenols six different lipases was initially screened for activity[275, 276]. Out of these, only the lipase from *Chromobacterium viscosum* (CVL) showed significant activity. In a subsequent study, the lipase from *Pseudomonas cepacia* (PSL) turned out to be a more efficient biocatalyst, which was succesfully used for the regioselective acylation of various aromatic dihydroxycarbonyl compounds[277], and (+)-catechin[278]. Thus, by using PSL as the biocatalyst the dihydroxy aldehydes and ketones **177**, **178** and related compounds were selectively acetylated in conversions ranging from 20 to 97% using vinyl acetate as the acyl donor (Fig. 18-24)[277]. (+)-Catechin **179** was also subjected to irreversible acyl transfer conditions. In this case, both the 5- and 7-monoacetates were obtained in 40% and 32% yield, respectively[278]. Interestingly, the inability of the lipase from *Aspergillus niger* to acylate aromatic hydroxy groups has consequently been used for the selective acylation of primary aliphatic hydroxy functions in molecules containing both aromatic and aliphatic OH-groups[279]. In fact, even PSL preferentially acylates primary aliphatic hydroxy groups if they are present in the compound[280].

In the deprotection of peracetylated polyphenolic compounds a somewhat different scheme has emerged. In this area, a broader spectrum of lipases has been used successfully. For example, the pentaacetyl derivative of catechine **179** was treated with PSL under alcoholysis conditions (THF, n-butanol) to give the 3,3',4'-trisacetate in 50% yield after 12 hours[278]. On longer exposure to the biocatalyst, the 3-monoacetyl derivative was isolated in 95% yield.

Thus, the coumarine **180**, the chromanone **181**, the chalcone **182**, the flavanone **183** as well as several flavones, e.g. **183** and **185** were regioselectively deacylated by employing different lipases in organic solvents (Fig. 18-24). Porcine pancreatic lipase (PPL) predominantly attacks one of the phenolic acetates present in **180**-**183** with

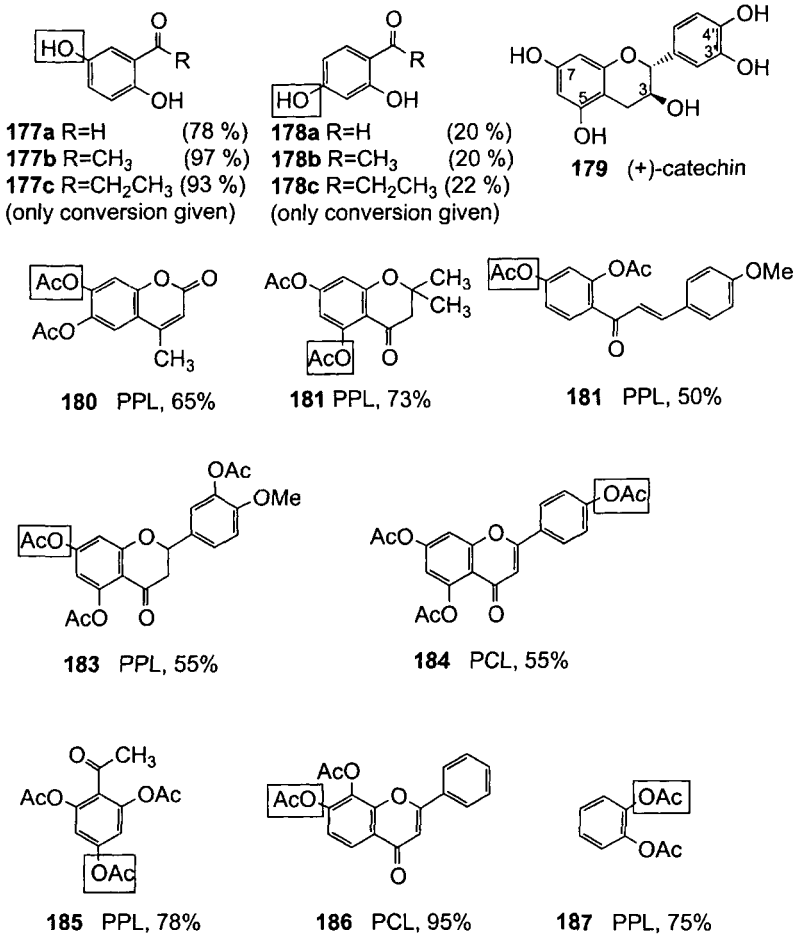


Figure 18-24. Selective enzymatic protection and deprotection of polyphenolic compounds.

good to high regioselectivity and produces the respective selectively protected compounds available in good yields [281–283]. The flavone acetates **184** and **186** can be partially deacylated with high regioselectivity by transesterification using lipase from *Pseudomonas cepacia* (PSL) and n-butanol in THF. [284,285] However, in other cases the positional specificity displayed by the enzyme was less pronounced. This technique has allowed for an efficient construction of a selectively *O*-methylated flavonoid [284].

In addition, aryl alkyl ketones which are important starting materials for the synthesis of polyphenolic natural products may be manipulated selectively by making use of an enzymatic saponification [283, 285–287]. In general, in these cases the sterically better accessible ester groups are cleaved, as for instance in **185** [285]. All of these examples have in common the fact that a carbonyl group is either directly or vinylogously attached to the aryl moiety. Without such a function present in the

molecule, the biocatalysts failed to differentiate the ester groups or completely deacylated the substrates. However, by using the lipases from porcine pancreas (PPL) or *Candida cylindracea* (CCL) immobilized on microemulsion-based gels it was possible to monodeacylate resorcinol and related diesters such as **187** in high yields[158]. Alternatively, by using *tert*-butyl methyl ether saturated with water as the solvent, it was possible to monodeacetylate diacetoxynaphthalenes selectively[288]. The influence of the solvent was exemplified by charging the solvent system to acetone/buffer: under such conditions only completely deacylated products were obtained.

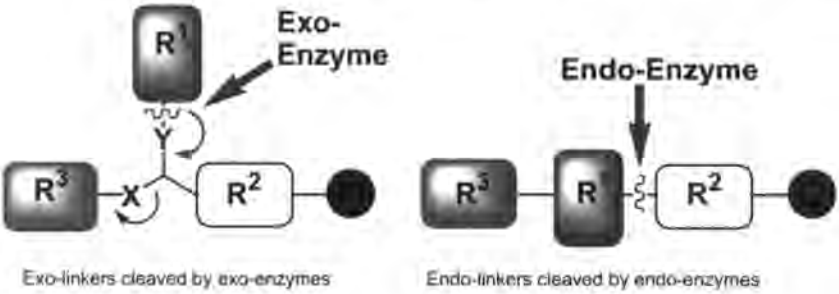
18.6
Biocatalysis in Polymer Supported Synthesis: Enzyme-labile Linker Groups

Combination chemistry and parallel synthesis of compound libraries on polymeric supports are efficient methods for the generation of new substances with a predetermined profile of properties[289–291]. The anchoring of one reactant to a polymeric support has the advantage that an excess of reagent may be used, while purification is kept manageable. This is particularly important if the reaction is to be carried out with several reactants in the same reaction vessel. Solid phase synthesis involves the use of linkers between the compounds to be varied combinationally and the solid supports which are stable during the reactions. These linkers have to be cleavable as desired, usually at the end of the synthetic sequence, with high selectivity and in good yield, without affecting the structure(s) of the product(s) that are released from the polymeric supports.

Linkers have previously usually been cleaved by classical chemical methods, for instance using strong acids. Such conditions often restrict the application of the linkers, i.e. acid-sensitive linkers are not suitable for acid-labile compounds, such as carbohydrates. Specific linkers have therefore been developed for acid-labile compounds, such as silylether linkages, thioether linkages[292], and ester linkages[293]. Although such linkers may be cleaved in the presence of acid-labile groups, they have the disadvantage that they are themselves quite labile to common chemical reagents that one might want to employ on the solid phase. For example, esters and silylethers are unstable to bases and thioethers are unstable in the presence of oxidants, such as *m*-chloroperbenzoic acid, and to electrophilic reagents, such as alkylating agents.

In principle linker groups are polymer-enlarged versions of blocking functions used in regular solution phase chemistry. Therefore, enzymatic transformations that may be employed for the removal of protecting groups in solution in principle may also open up alternative opportunities for releasing compounds from polymeric supports. The linkers developed so far can be divided into exo- and endo-linkers (Fig. 18-25) cleavable by exo- endo-enzymes, respectively, as proposed by Flitsch et al.[294].

Exo-linkers are composed of three units: (i) a group providing the site for enzyme catalyzed hydrolysis (R^1); (ii) a site for attachment of the target molecule (R^3); and (iii) a site for attachment to a further optional spacer (R^2).



R^1: group providing the site for enzyme catalyzed hydrolysis,
R^2: optional intermediate linked to a solid support,
R^3: residue to be synthesized and varied in the course of a synthesis on the support,
X: O, N(H), N(R"), C(O)O, S, C(O)N(H) or C(O)N(R"),
R" is a noninterfering substituent, Y: O or NH.

Figure 18-25. Graphical representation of exo- and endo-linkers.

Endo-linkers are linkers in which the target molecule (R^3), the group, which provides a site for enzyme catalyzed hydrolysis (R^1) and a further optional spacer (R^2) are attached to the polymeric support in a linear arrangement. By means of enzyme mediated dissection, the target molecule, in many cases tagged with the functional group recognized by the enzyme, is released.

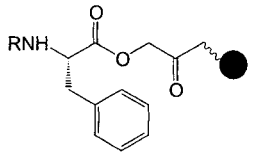
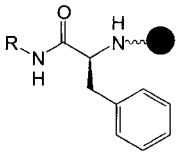
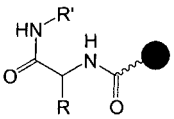
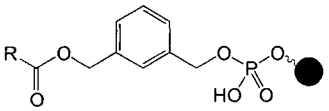
Examples of endo-cleavable linkers have been reported (Table 18-5). However, in many cases the product is tagged with part of the linker. For instance, the endo-peptidase chymotrypsin cleaves endo-linkers towards the middle of a peptide-chain or "internally". Not only does this limit the methodology to a very small number of enzymes, but it may also restrict the structure of molecules that can be generated. For instance, this method will typically (but not necessarily, see Figs. 18-26 and 18-27) generate compounds containing C-terminal aromatic amino acids, which are necessary for recognition by chymotrypsin. By contrast, exo-linkers do not restrict the structure of the reactant and can be cleaved by more readily available exo-enzymes, which act at the end of a chain or "externally" (Table 18-5). Furthermore exo-cleavable linkers yield untagged products upon cleavage from the solid support.

18.6.1
Endo-linkers

For a better overview, examples of endo-linkers and the enzymes used for the cleavage of the product from the solid phase which have been described in the literature so far are given in Table 18-5.

Wong and coworkers[295] introduced a silica-based solid support with a specific enzymatically cleavable linker for the synthesis of glycopeptides and oligosaccharides. They found that styrene- and sugar-based polymers tend to swell which leads to a low coupling yield. Their choice of solid support is aminopropyl silica based on the

Table 18-5. Examples of endo-linkers and the appropriate cleavage enzymes.

Linker	Enzyme	Examples	Ref.
	α-Chymotrypsin	Glycopeptide synthesis	[296]
	α-Chymotrypsin	Oligosaccharide synthesis	[297–298]
	Ceramide glycanase	Oligosaccharide synthesis	[299]
	Phosphodiesterase	Peptide synthesis	[301]

facts that: (a) it is compatible with both aqueous and organic solvents, (b) it has a large surface area accessible to biomolecules, and (c) it has sufficient density of functional groups.

A hexaglycine spacer was attached to the solid support to give a substitution of 0.2 mmol g^{-1} of dry silica and the excess amino groups were then capped using acetic anhydride. In the next step a selectively cleavable, α-chymotrypsin sensitive, phenylalanine ester **189** was implemented for the release of the products from the solid support under mild conditions. Then it was transformed to **190** followed by reactions with glycosyl transferases to yield **191**. Finally, the desired glycopeptide was cleaved from the solid support in high yield by treatment of **191** with α-chymotrypsin (Fig. 18-26).

Nishimura and coworkers[296–297] described a novel method for the enzymatic synthesis of oligosaccharide derivatives employing an α-chymotrypsin sensitive linker. The synthesis of the water soluble GlcNAc-polymer **197**, sensitive to α-chymotrypsin, is shown in Fig. 18-27. Oxazoline derivative **193** was coupled with 6-(*N*-benzyloxycarbonyl-L-phenylalanyl)-amino-hexanol-1 (**194**) followed by *N*-deprotection of the phenylalanine and subsequent condensation with 6-acrylamido caproic acid **195**. De-*O*-acetylation gave the polymerizable GlcNAc derivative **196**. Finally, copolymerization of acrylamide and monomer **196** in the presence of ammoniumpersulphate (APS) and *N,N,N',N'*-tetramethyl ethylene diamine (TMEDA) gave the

Figure 18-26. Glycopeptide synthesis and α-chymotrypsin catalyzed release from the solid support.

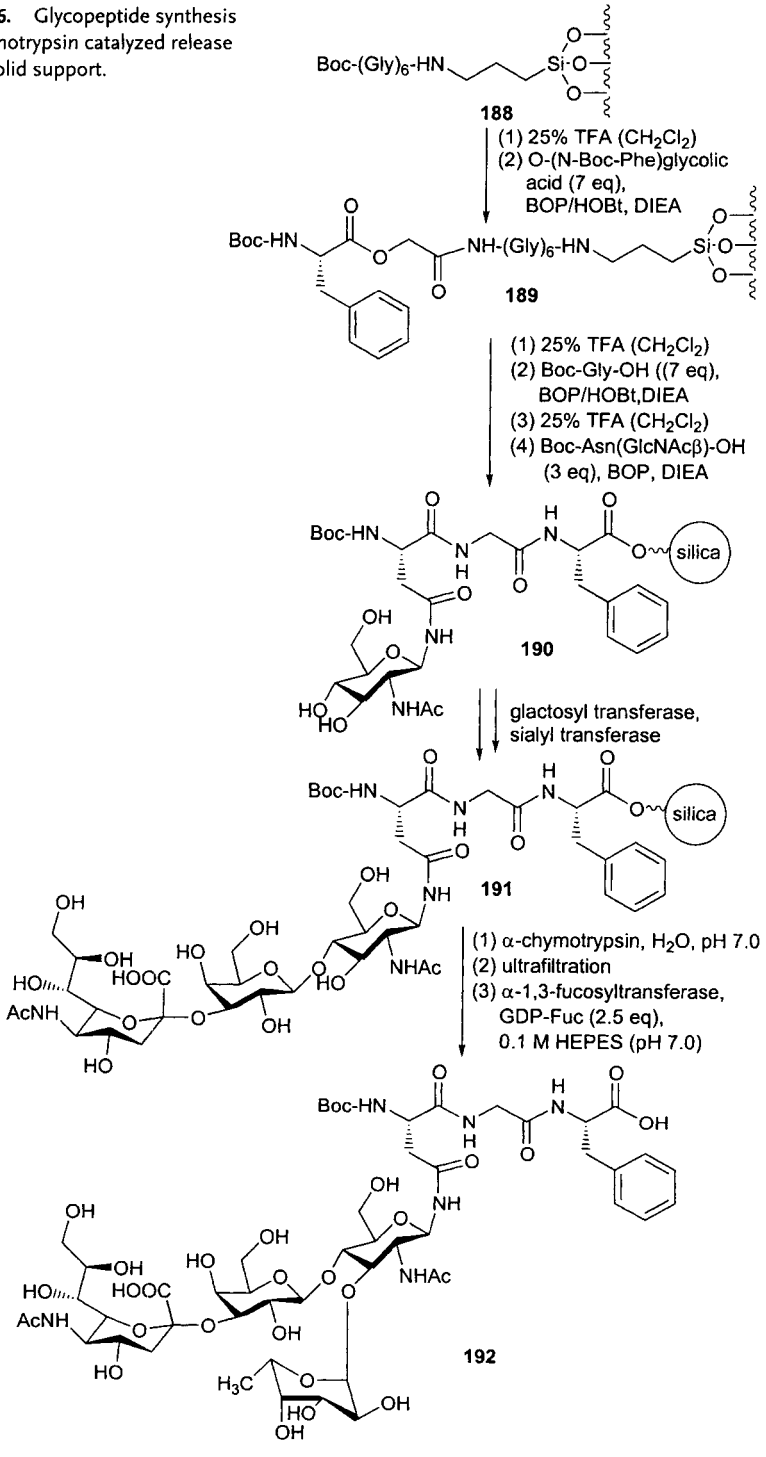
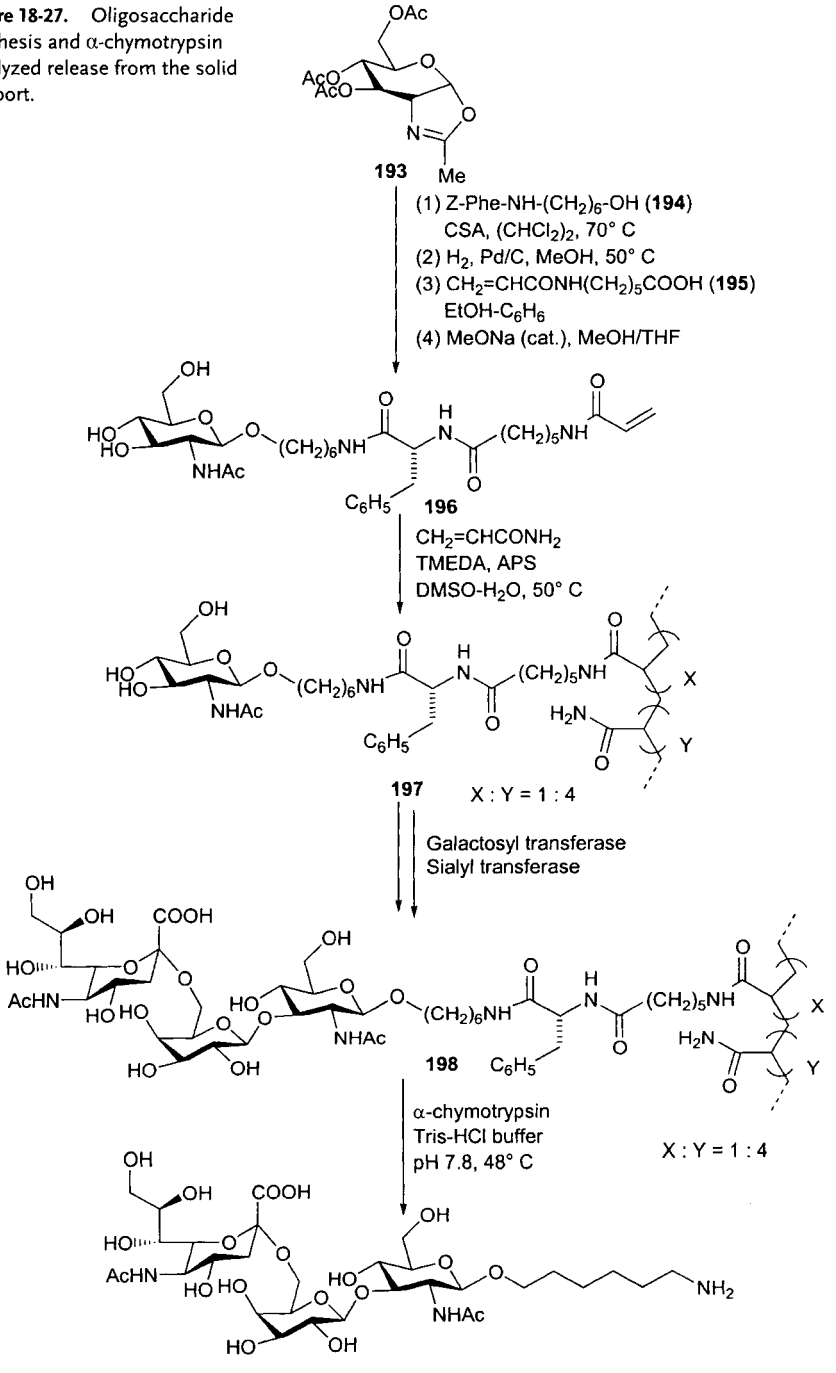


Figure 18-27. Oligosaccharide synthesis and α-chymotrypsin catalyzed release from the solid support.



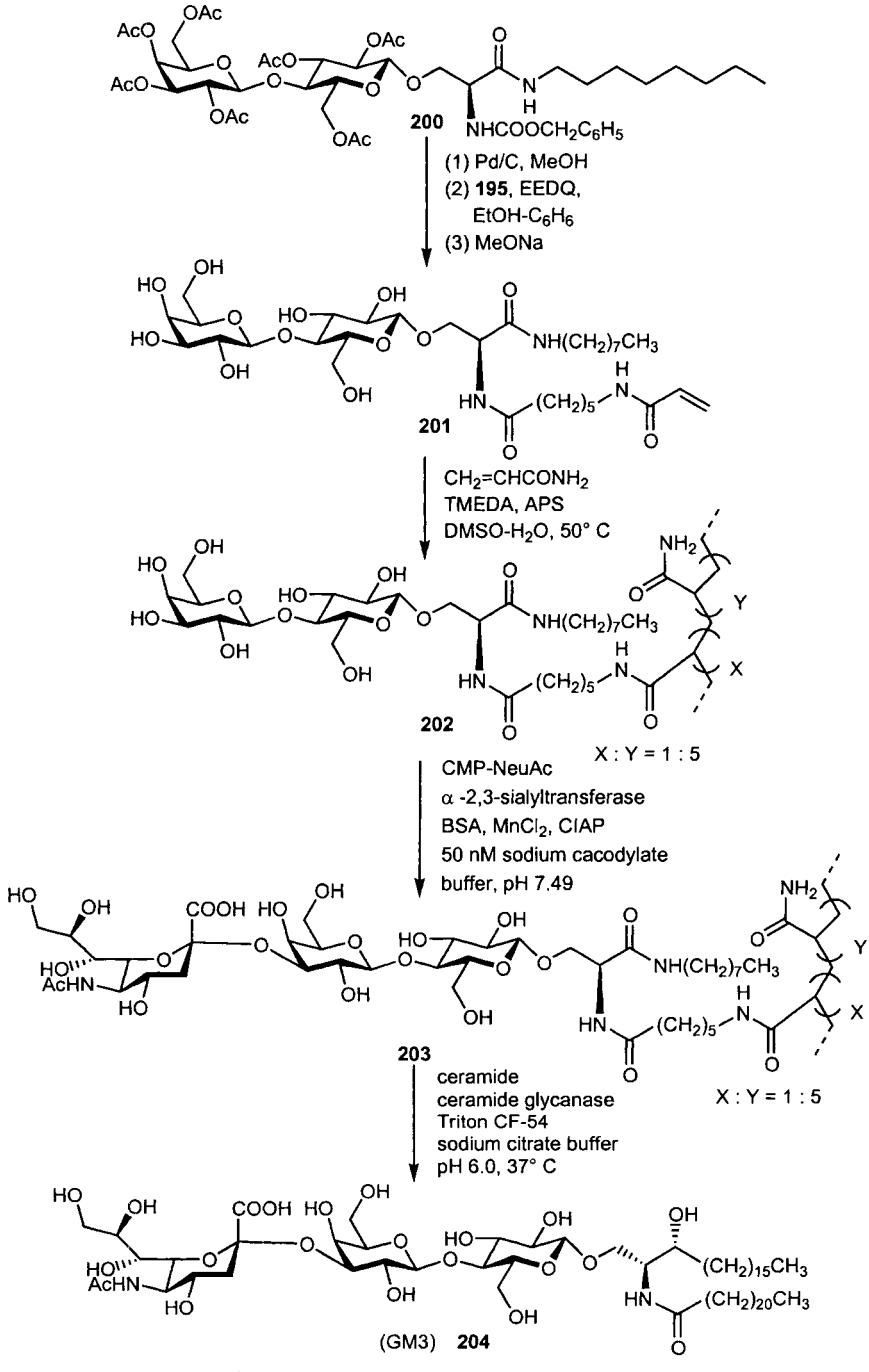


Figure 18-28. Ceramide glycanase mediated release by transglycosylation.

polymer **197** in high yield. The polymer **197** was then subjected to galactosylation and subsequent sialylation with the corresponding glycosyl transferases to yield **198**. The final product **199** was cleaved from the water-soluble support by treatment with α-chymotrypsin at 40 °C for 24 h in 72% overall yield from **197**.

Nishimura and Yamada[298] introduced a water-soluble polymeric support having a linker recognized by ceramide glycanase for a synthesis of ganglioside GM3 (**204**). Synthesis of the polymerizable lactose derivative **201** with a ceramide glycanase sensitive linker is shown in Fig. 18-28. The lactosyl ceramide (LacCer) mimetic glycopolymer **202** is obtained from the monomeric precursor **201** by co-polymerization with acrylamide.

This solid support **202** was converted into the intermediate product **203** by sialylation using βGal1→3/4GlcNAc α-2,3-sialyltransferase. Finally, the polymeric support was cleaved by transglycosylation with leech ceramide glycanase in the presence of excess ceramide as the acceptor to give the desired product **204** in high yield (Fig. 18-28). An advantage of the water-soluble polymer is that the transformation can be monitored by NMR spectroscopy during the enzymatic glycosylation steps.

Arrays of up to 1000 peptide nucleic acid (PNA) oligomers of different sequence were synthesized by Jensen et al. on polymer membranes (Fig. 18-29)[299]. The PNA chain was linked to the peptide spacer glutamic acid-(γ-*tert*-butyl ester)-(ε-aminohexanoic acid)-(ε-aminohexanoic acid) (Glu[OtBu]-εAhx-εAhx) via an enzymatically cleavable Glu-Lys handle. The Glu[OtBu]-εAhx-εAhx spacer was coupled to the amino-functionalized membrane by standard Fmoc-Chemistry. Then the membranes were mounted in an ASP 222 Automated SPOT Robot and a grid of the desired format was dispensed at each position. The free amino groups outside the spotted areas were capped and further chain elongation was performed with Fmoc-protected PNA monomers to synthesize the desired PNA oligomers. After completion of the synthesis, the PNA oligomers were cleaved from the solid support by incubation with bovine trypsin solution in ammonium bicarbonate at 37 °C for 3 h.

One of the very first papers concerning endo-linkers was published by Elmore et al. (Fig. 18-30)[300]. They described a new linker containing a phosphodiester group for solid phase peptide synthesis using a Pepsyn K (polyacrylamide) resin. After completion of coupling and deprotection cycles, the phosphodiester **207** was cleaved with a phosphodiesterase. In this way β-casomorphin, Leu-enkephalin and a col-

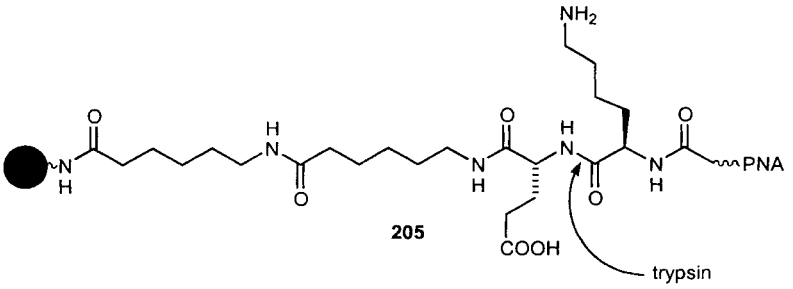


Figure 18-29. Trypsin mediated cleavage of a peptide bond in PNA oligomer synthesis.

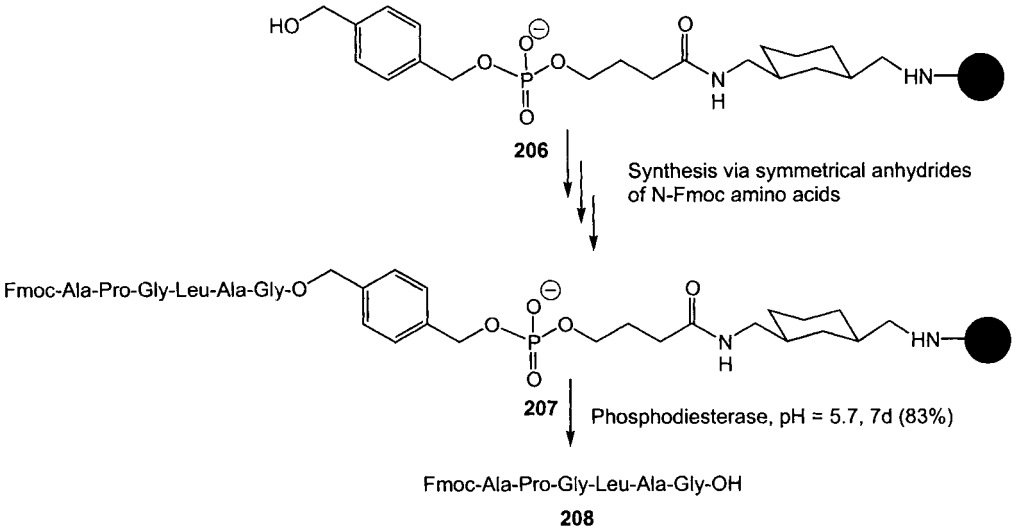


Figure 18-30. Synthesis of a collagenase substrate on a phosphodiesterase-scissile linker.

lagenase substrate were synthesised in high yields. In the context of enzymatic cleavage of linkers on polymeric supports particular attention was paid to the general question of whether enzymatic transformations on resins are viable and high yielding. An in-depth treatment of this problem is beyond the scope of this review. However, a few examples for the application of biocatalyzed transformations on solid supports will serve to illustrate that such transformations can indeed be employed advantageously for various purposes.

Meldal et al. described the proteolytic cleavage of the alanine-tyrosine bond in a resin-bound decapeptide by treatment with the 27 kDa protease subtilisin BNP' to demonstrate the accessibility of the interior of the newly designed SPOCC-resin[301] to enzymes[302].

Furthermore, enzymatic hydrolysis of model isopeptides N^{ε}-oligo(L-methionyl)-L-lysine from Bio-beads[303] by pepsin, chymotrypsin, cathepsin C (dipeptidyl peptidase IV) and intestinal aminopeptidase N was investigated using high-performance liquid chromatography to identify and quantify the hydrolysis products[304].

Larsen et al. reported the enzymatic cleavage of a desB30 insulin B-chain from a presequence $(Lys(Boc))_6$. This spacer shifts the conformation of the growing peptide chain from a β-structure to a random coil conformation and reduces peptide-chain aggregation, which otherwise causes serious synthetic problems. Novasyn KA-[305] was employed as a solid support, but unfortunately, no information about the enzyme used was reported[306].

Barany et al. were the first to exploit the different enzyme accessibilities of surface and interior areas of a given bead and the resulting differentiated bead was used to synthesize a peptide library on the surface and the code for this on the interior simultaneously[307]. This clever strategy is illustrated in Fig. 18-31. Selective cleavage of short N^{α}-protected peptide substrates with chymotrypsin from the surface area of

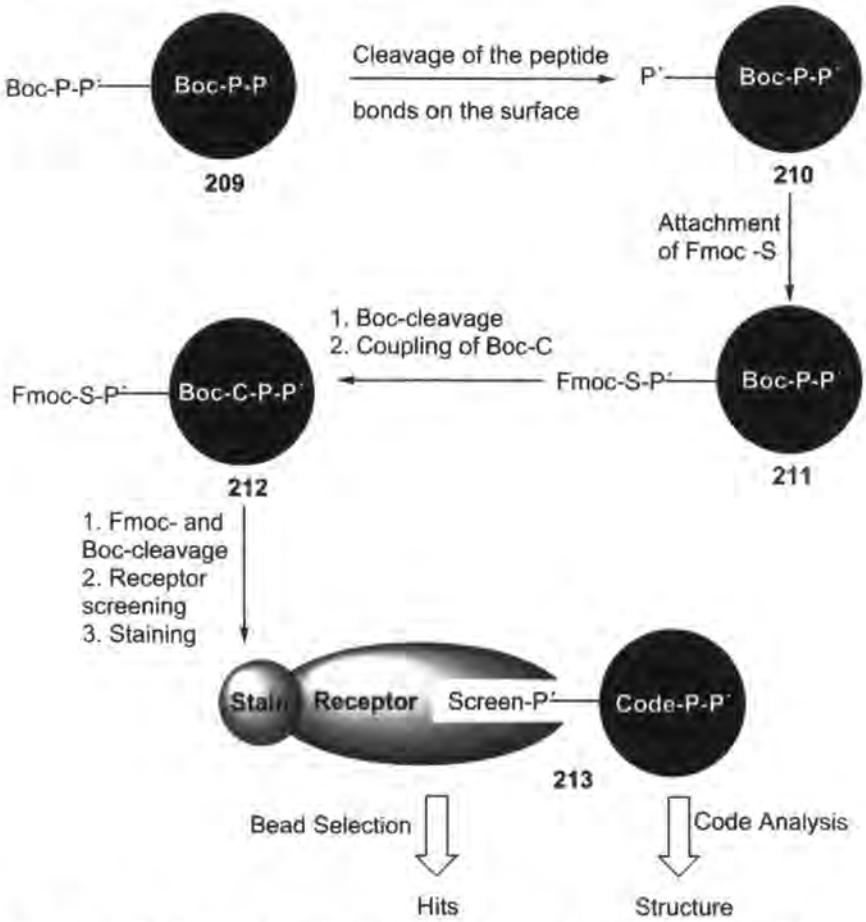


Figure 18-31. Peptide encoded combinatorial peptide libraries via enzyme-mediated spatial segregation. P-P': substrate with a scissile bond between P and P'; S: terminal residue of the screening structure, C: terminal residue of the coding structure.

TentaGel-AM-beads **209** leaves the majority of the peptide attachment sites in the interior uncleaved to afford **210** ("shaving" methology). The first residue is attached using orthogonal FMOC-chemistry to provide **211**. Coding is done by using standard BOC-chemistry on the interior of the bead to yield **212**. Repetition of this process furnishes a surface peptide, which is encoded internally (**213**).

This generation of two structures on the same bead allowed the investigation of the synthesized peptide library (1×10^5 members) with different receptors (anti-β-endorphin antibody, streptavidin and thrombin). After the staining procedure had been carried out, the beads that showed a color were selected for sequencing and the coding peptides present within the bead were used to deduce the binding structures. This screening led to the discovery of a new thrombin ligand, which binds with an affinity one order of magnitude higher than the natural motif.

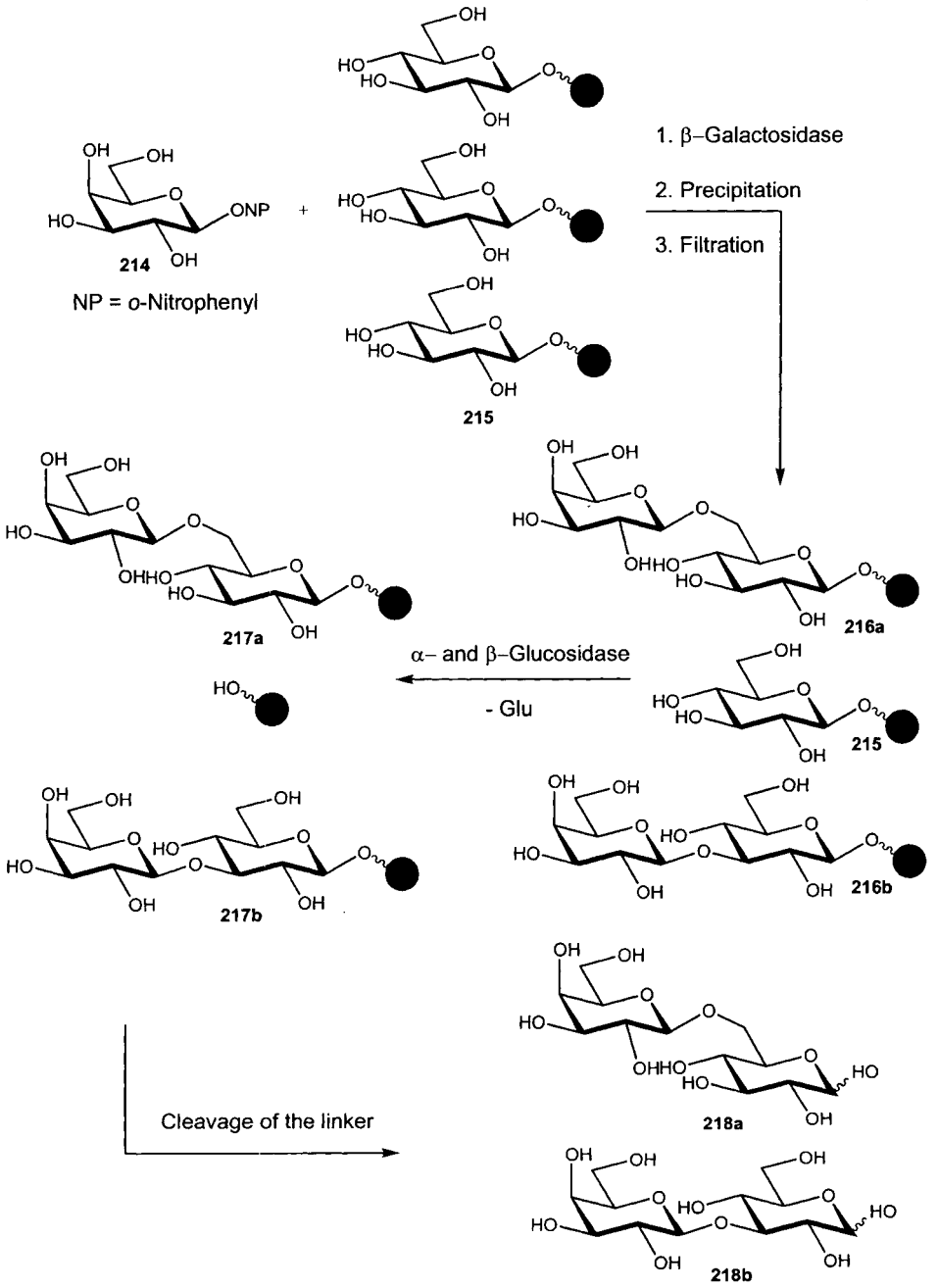


Figure 18-32. General strategy for the liquid-phase synthesis of disaccharides using glycosidases.

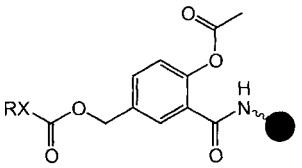
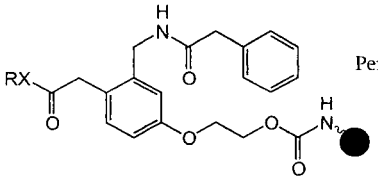
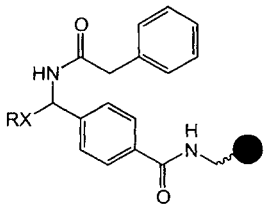
Fernandez-Mayoralas and coworkers[308] used the high substrate specifity of enzymes in their synthesis of galactose-glucose-disaccharides (**218**) on an MPEG-support[309]. After galactosylation of glucose immobilized on the soluble support (**215**) using β-galactosidase, the unreacted monosaccharide glucose was removed by the combined use of α- and β-glucosidases to obtain only MPEG-bound disaccharides (**216**, Fig. 18-32). Finally the disaccharides **218** obtained were released from the support by ethanolysis.

Schmitz and Reetz described the solid phase enzymatic synthesis of oligonucleotides on Kieselguhr-PDMA-resins via T4 RNA ligase. Concomitantly, they found that RNase A selectively cleaves the last bound nucleotide at the ribose sugar leaving a 3',5'- diphosphorylated oligomer behind on the resin, but application in actual synthesis has not yet been undertaken[310].

18.6.2
Exo-linkers

An exo-linker according to Fig. 18-25 must contain an enzyme labile group R^1, which is recognized and attacked by the biocatalyst. Possible combinations could be: phenylacetamide/penicillin amidase, ester/esterase, monosaccharide/glycosidase,

Table 18-6. Examples of exo-linkers and the appropriate cleavage enzymes.

Linker	Enzyme	Examples	Ref.
	Lipase	Picet-Spengler reaction, nucleoside immobilization	[313]
	Penicillin acylase	Palladium cat. C-C-couplings, Mitsunobu- and Diels-Alder reactions, 1,3-dipolar cycloadditions	[318, 319]
	Penicillin acylase	Immobilization of alcohols (e.g. Fmoc protected serine methyl ester, glycosides) and amines (e.g. phenylalanine)	[312, 313]

phosphate/phosphatase, sulfate/sulfatase and peptides/peptidases[311]. Up till now only the following systems have been worked out (Table 18-6).

In independent and simultaneous investigations Flitsch and coworkers [311, 312] and Waldmann and coworkers[313, 314] developed a selectively cleavable exo-linker, which can be cleaved with penicillin G acylase, a commercially available and widely used enzyme[77].

Penicillin acylase catalyzes the hydrolysis of phenylacetamides and has been used in peptide synthesis for the cleavage of protecting groups[6, 315]. In linker **219** developed by Flitsch and coworkers[311, 312] (Fig. 18-33) -XR represents the alcohol or amine group of the target molecule. Hydrolysis of the phenylacetamide moiety generates the hemiaminal **221** which readily fragments in an aqueous medium and thereby releases the desired products, RXH. The thioethyl group present in the anchor group of **219** was activated by treatment with *N*-iodosuccinimide (NIS) followed by displacement with a variety of alcohols (**223–225**). To prove the possible application of this linker in solid phase carbohydrate synthesis protected glycosides **226** and **227** were coupled to linker **219** and released enzymatically. Flitsch et al. also described the immobilization and enzymatic cleavage on a variety of amines[311]. Nevertheless, the application of this enzyme-labile linker group in multi-step syntheses on the solid phase and subsequent enzyme-initiated release from the polymeric support has not been described yet.

Waldmann and coworkers described designed exo-linker **228**[313, 314]. The anchor group comprises a 4-acyloxy-3-carboxybenzyloxy group, which is recognized and attacked by the biocatalyst, so that a spontaneously fragmenting intermediate is generated, thereby releasing the desired compound (Fig. 18-34)[53, 54, 57]. The linker **228** is attached as an amide to the solid phase. Cleavage of the acyl group by a lipase generated a phenolate **229**, which fragments to give a quinone methide **230** and releases the product **231**. The quinone methide remains on the solid phase and is trapped by water or an additional nucleophile.

Following on from this cleavage principle, amines (bound as urethanes), alcohols (bound as carbonates), and carboxylic acids (bound as esters) can be detached from the polymeric carrier. The substrate specificity of the enzyme guarantees that only the intended ester is cleaved. TentaGelS-NH_2 was chosen as the polymeric support, i.e. a polystyrene resin equipped with terminally NH_2-functionalized oligoethylene-glycol units. It has a polar surface and swells in aqueous solutions allowing the biocatalyst access to the polymer matrix[316].

The applicability of the enzyme-labile anchor group was demonstrated by the synthesis of tetrahydro-β-carbolins **237** employing the Pictet-Spengler reaction (Figure 18-35). The benzylic alcohol group of the linker **232** was first esterified with Boc-L-tryptophan, and after its *N*-terminal deprotection the support-bound tryptophan **233** was reacted with aliphatic and aromatic aldehydes to give imines **234**, which cyclized immediately in reasonable to high yields to the tetrahydro-β-carbolins **235**. Lipase RB 001–05 selectively attacked the acetate incorporated into the linker and generated the corresponding phenolate **236**, which then fragmented spontaneously. Following these multistep transformations the desired tetrahydro-β-carbolins **237** were obtained in 70–80% yield.

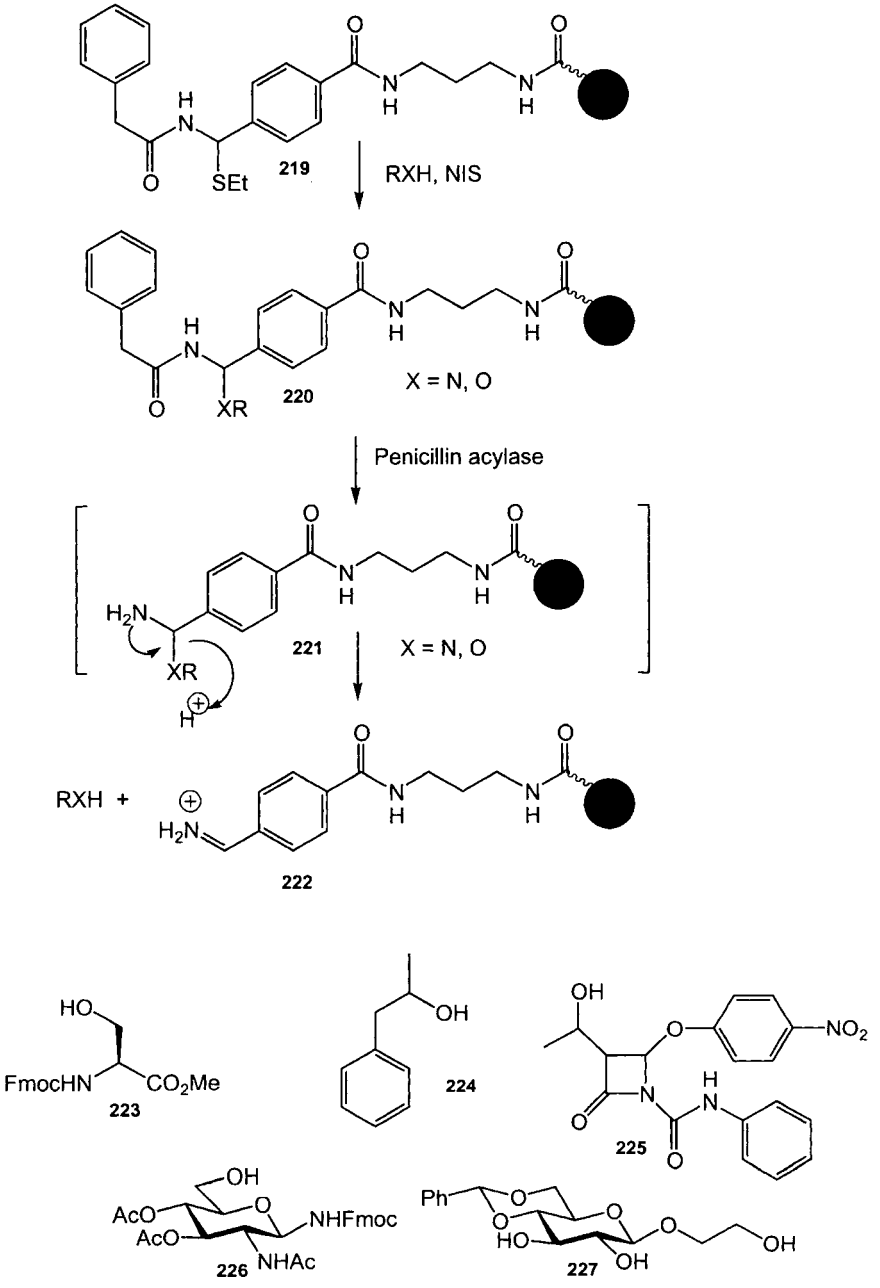


Figure 18-33. Loading and cleavage of a penicillin acylase scissile linker.

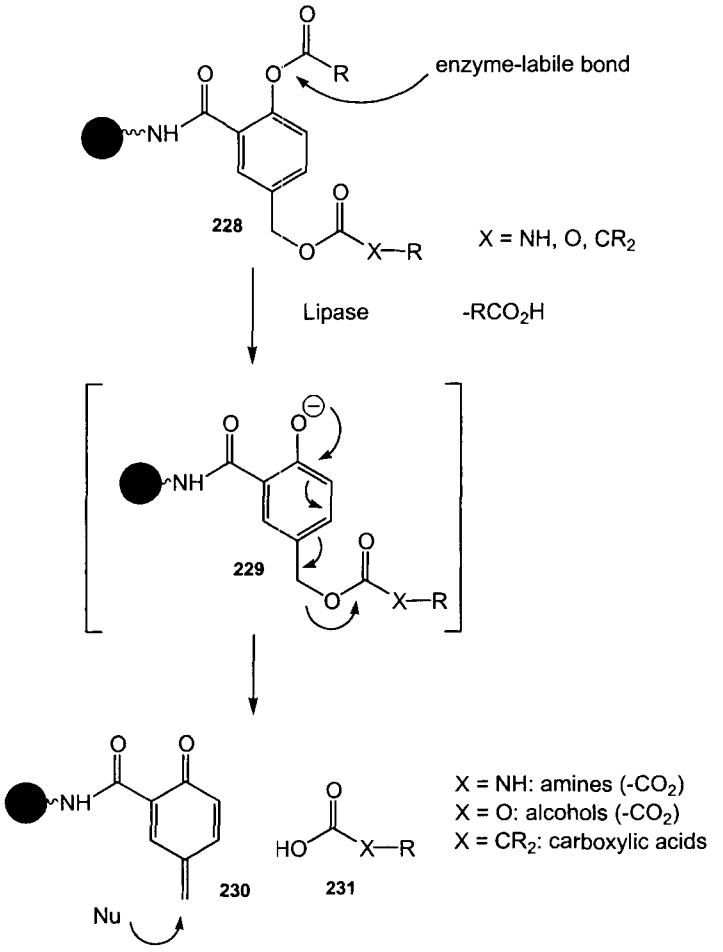


Figure 18-34. Principle for the development of the enzyme-labile 4-acyloxy-benzyloxy linker group.

Waldmann and coworkers developed a second exo-linker following a new approach[317, 318] which makes use of a safety-catch linker. It is based on the enzymatic cleavage of a functional group embodied in the linker. In this way an intermediate is generated, which subsequently cyclizes intramolecularly according to the principle of assisted removal[2, 319–322] and thereby releases the desired target compounds (Fig. 18-36). The linker group is immobilized as a urethane on the amino-functionalized carrier **238**. It facilitates the attachment of a variety of molecules such as alkyl halides, alcohols or amines bound as carboxylic acid esters and amides. According to the safety-catch principle, the separation of the desired products proceeds in a two-step process. First, penicillin G acylase hydrolyzes the phenylacetamide with complete chemo- and regioselectivity, under exceptionally mild conditions (pH 7.0, room temperature or 37 °C)[30, 72, 74]. Then the activated intermediate generated,

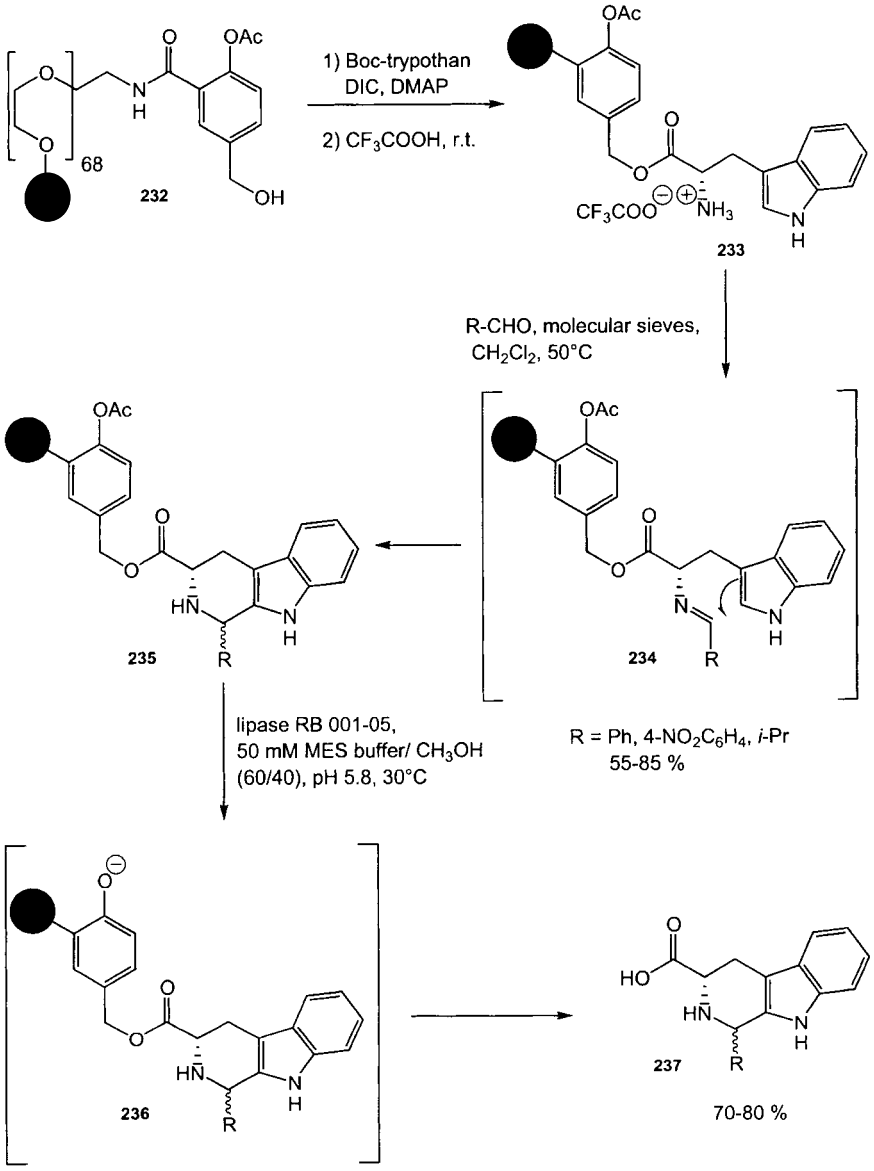


Figure 18-35. Solid phase synthesis of tetrahydro-β-carbolins and subsequent detachment by enzyme initiated fragmentation of the anchor group.

i. e. benzylamine **239**, cyclizes to polymer-bound lactam **240** and releases the desired target molecule **241**.

POE 6000 was used as the polymeric support, a soluble polyethyleneglycol derivative functionalized at both termini with an amino group and with an average molecular mass of 6000 Da [323–324]. After completion of the homogeneous reactions

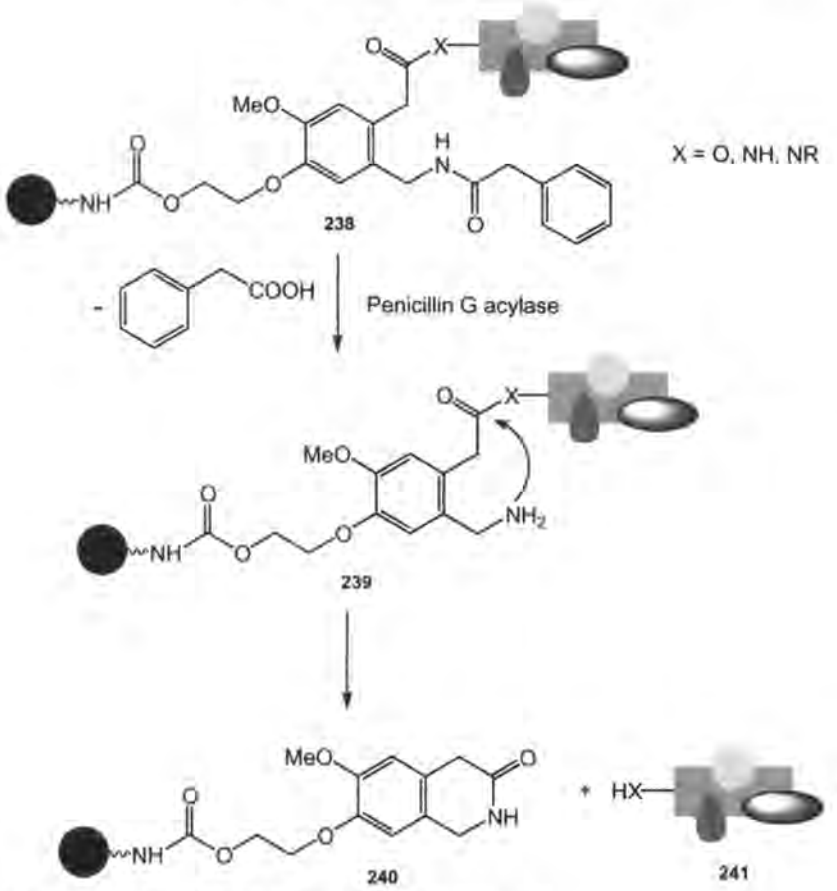


Figure 18-36. Principle of the enzyme-labile safety catch linker.

it can be precipitated, filtered off, and washed with diethyl ether, thereby facilitating the separation of surplus reagents and the side products. Furthermore it allows for NMR spectroscopic monitoring of the reactions[325]. Most importantly, it is soluble in aqueous solutions, thereby allowing efficient access of the enzyme to the polymer-fixed linker group.

The suitability of the polymer-linker conjugate was examined for a variety of transformations, in particular Pd^0-catalyzed reactions. For instance, the polymer-bound aryl iodide **242** was transformed quantitatively in a Heck reaction to a cinnamic acid ester **243** and to biphenyl **245** in a Suzuki reaction. It gave an alkine **244** in a Sonogashira reaction (Fig. 18-37). The desired benzyl alcohols **246–248** were released by incubation of the corresponding polymer conjugates **243–245** with penicillin G acylase at pH 7 and 37 °C in high yields and isolated with a purity of >95 % by simple extraction with diethyl ether.

Furthermore, the applicability in a Mitsunobu esterification reaction and a Diels-Alder reaction was proven (Fig. 18-38). The polymer-bound benzyl alcohol **249** was

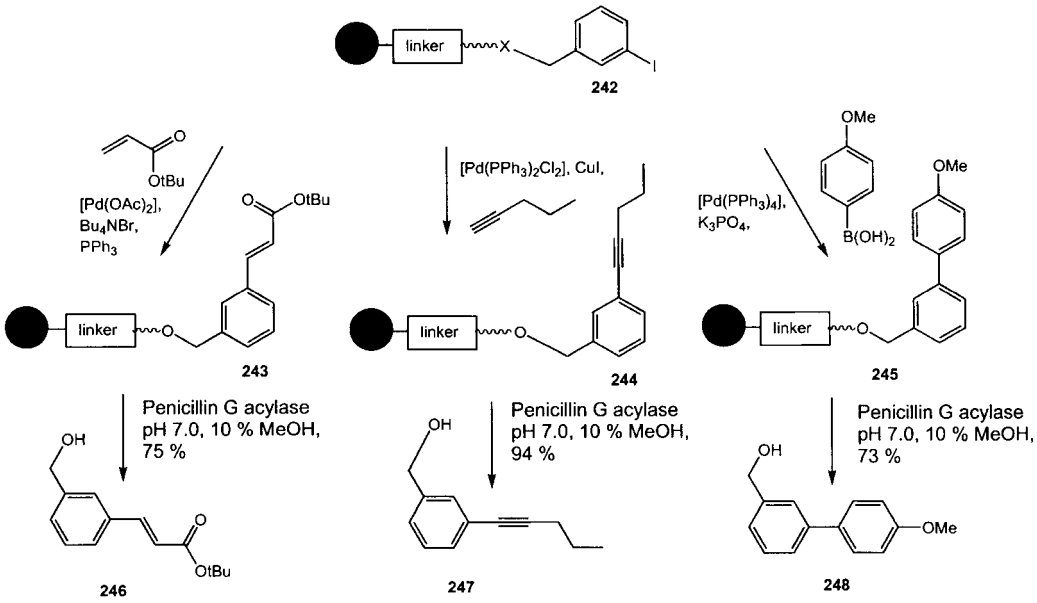


Figure 18-37. Pd^0-catalyzed reactions on enzyme labile linker-conjugates.

reacted with 4-acetamidophenol in the presence of the Mitsunobu reagent to give phenyl ether **250** in quantitative yield. It was released from the polymeric support in high yield. For the Diels-Alder reaction, polymer-bound acrylic acid ester **252** was treated with cyclopentadiene. The cycloaddition product **253** was formed with an endo/exo ratio of 2.5 : 1 and with quantitative conversion. The subsequent enzymatic release delivered the corresponding alcohol (**251**, **254**) in high yield and purity.

18.7 Outlook

During recent decades substantial progress was achieved in the development of enzymatic protecting group techniques. In particular, it was demonstrated that these methods offer viable alternatives to classical chemical approaches. Not only do the biocatalyzed transformations complement the arsenal of chemically removable protecting groups, but in many cases they additionally offer the opportunity to carry out useful functional group interconversions with selectivities which can not or only barely be matched by chemical techniques. However, the overwhelming majority of the investigations carried out in this area has restricted themselves to the study of the protection and deprotection of model compounds. Complex synthetic schemes were nearly always avoided. Whereas this appears to be particularly true in the area of carbohydrates, noticeable examples which demonstrate the capacity of these biocatalyzed processes were recorded in peptide and peptide conjugate chemistry, i. e. in

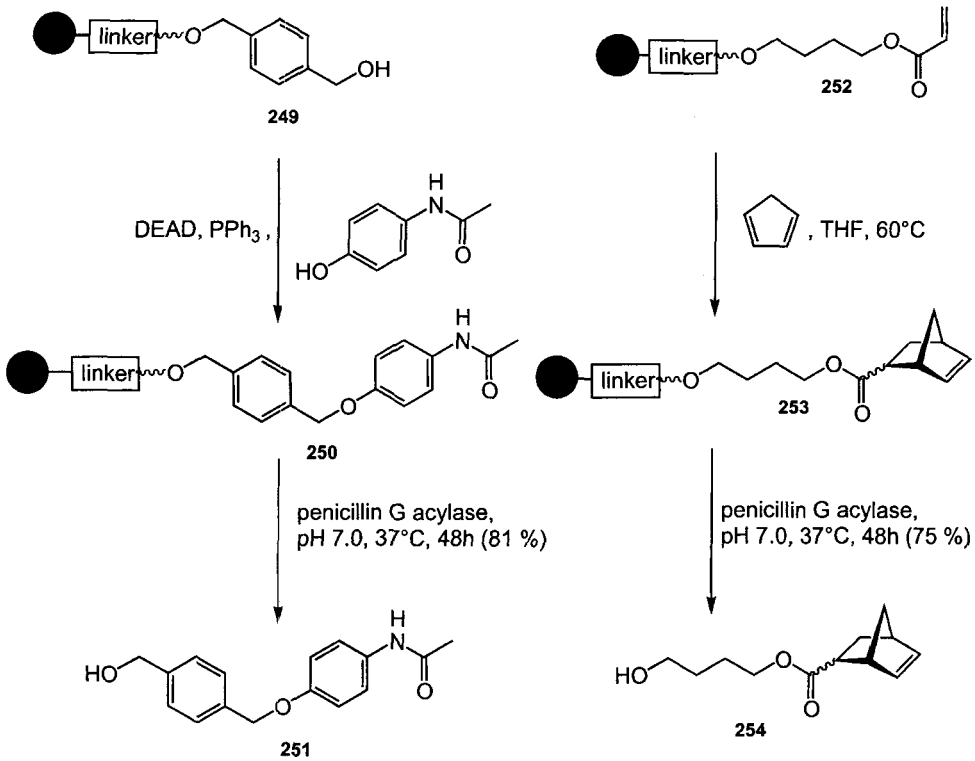


Figure 18-38. Mitsunobu and Diels-Alder reaction on enzyme labile linker-conjugates.

the synthesis of lipo-, glyco and nucleopeptides. The data and observations highlighted above, however, provide a solid basis for the application of biocatalysts in the handling of protecting group problems in complex multistep syntheses.

On the other hand, the use of biocatalysts in protecting group chemistry in the sense of a general method deserves and is certainly awaiting further intensive development. Numerous applications of the known enzymes appear to be possible in all areas of preparative chemistry. In addition, the use of catalytic proteins which have not yet been applied to carry out protecting group manipulations and of biocatalysts unknown today or which will be developed in the future, e.g. by evolutionary approaches, will create new opportunities for improved organic syntheses.

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19
Replacing Chemical Steps by Biotransformations: Industrial Application and Processes Using Biocatalysis

Andreas Liese

'Bacteria are capable of bringing about chemical reactions of amazing variety and sublety in an extremely short time ... Many bacteria are of very great importance to industry where they perform tasks which would take much time and trouble by ordinary chemical methods.'

Sir Cyril Hinshelwood, 1956[1]

19.1
Introduction

Starting with big promises and many expectations in the seventies biocatalytic processes have left the status of a lab curiosity together with many prejudices far behind and are now established on an industrial scale[2]. Product examples range from amino acids, sugars, chiral alcohols and amines, and highly functionalized building blocks for pharmaceuticals to bulk chemicals such as acrylamide or propane-1,3-diol. When speaking about biotechnological processes one has to distinguish between fermentation processes and biotransformations. In a fermentation process the desired product is synthesized from nutrients and trace elements by either microorganisms (bacteria, yeasts, fungi) or higher cells such as mammalian or plant cells. The phrase "biotransformation" or "biocatalysis" is commonly used to describe a one-step or multi-step transformation of a precursor to the desired product using whole cells and/or (partly) purified enzymes. Whole cell processes are often used for redox reactions using the metabolism of the living cell for cofactor regeneration. In some cases the cell is used as a compartment containing the enzymes in a confinement allowing easier separation of the entire biocatalyst using centrifugation or microfiltration. If one has to deal with membrane-bound enzymes, whole cell biocatalysts are to be preferred.

Numerous authors have given overviews over biotransformations used in industry[3–11]. A very recent monograph summarizes almost 100 processes including many details on reaction conditions, screening of the biocatalyst or the product application[2]. The use of biocatalysis from the viewpoint of a chemist in the laboratory is also summarized in several books. Recent ones are[12–14].

In this contribution we shall focus on those examples where the biocatalytic step has distinct advantages over the corresponding chemical method, or even has replaced or is about to replace other methods. The reasons may be better regio-, stereo- or chemoselectivity, better product purity or simplified downstream processing. Often the incorporation of biocatalytic steps reduces the amount or toxicity of waste.

19.2 Types and Handling of Biocatalysts

This chapter tries to give a brief introduction to the types of biocatalysts, their requirements and methods of handling them. A more detailed treatment can be found in other chapters of this book or in the literature[15–17].

The biocatalyst may be a whole cell or a partly purified enzyme. In the first case the cell may be regarded as a mini-reactor with all necessary cofactors and enzymes to catalyze multiple steps concentrated in one cell. In the second case the main catalytically active species is isolated and purified.

For the whole cell systems either prokaryotic cells such as *Escherichia coli* or eukaryotic cells such as *Saccharomyces cerevisiae* or *Zymomonas mobilis* are used. Prokaryotic cells do not posses a nucleus. The nuclear material is contained in the cytoplasm of the cell. Therefore introduction and processing of foreign DNA to obtain a genetically engineered strain is simple. They are relatively small in size (0.2–10 μm) and exist mostly as single cells. Eukaryotic cells are higher microorganisms and have a true nucleus separated by a nuclear membrane. They are larger in size (5–30 μm) and sometimes form more complex structures. For both types the bioreactor has to fulfill certain requirements. An adequate supply of nutrients as well as oxygen into the bioreactor has to be assured. Parameters such as pH, oxygen, feed rate and temperature in the bioreactor must be kept within certain limits in order to guarantee optimum growth and/or metabolic activity of the cells. Especially when recombinant microorganisms are employed genetic stability during cultivation has to be observed carefully. Substrates, products and/or solvents required may be toxic for the cells and may therefore have to be added in low amounts to secure a low stationary concentration. The example of a ketone reduction with whole cells of *Zygosaccharomyces rouxii* shows one possible solution. The toxic substrate is adsorbed on XAD-7 resin (80 g/L resin, resulting in a concentration of 40 g/L reaction volume), and the resin is added to the fermentation broth. The equilibrium concentration in the aqueous phase is approximately 2 g/L. The product is adsorbed on the resin as well, thus providing integrated downstream processing[18–19].

When purified enzymes are used, basically the same requirements have to be met. The purification may cause additional costs, but contrary to a biochemical characterization it is not necessary to purify the protein to homogeneity. On the contrary, the remaining protein content in a partly purified enzyme may increase its stability. The only requirement is to have a functional pure enzyme, meaning that activities

catalyzing undesired side reactions have to be absent. This is the major advantage of purified enzymes over whole cell processes: side reactions may be easily avoided, and substrates that are toxic for the cell or which may not be able to enter the cell can be converted. For enzymes the thermal deactivation or deactivation by interphases (liquid-liquid, liquid-gas) may be limiting.

For industrial biotransformations, catalyst recovery and reuse are major issues. This may be desirable either for reasons of downstream processing or for repeated use in order to reduce the specific catalyst costs per kg of product produced. A very simple method is the use of membrane filtration. Because of the increasing number of membranes from different materials (polymers, metal or ceramics) this is an attractive alternative. Whereas for whole cells microfiltration or centrifugation can be applied, for the recovery of soluble enzymes ultrafiltration membranes have to be used[20–22]. Often immobilization on a support is chosen to increase the catalyst's stability as well as to facilitate its recovery. The main advantages of immobilization are:

- easy separation,
- often increased stability,
- use of fixed or fluidized bed reactors (for continuous processes).

Disadvantages are:

- loss of absolute activity due to the immobilization process,
- mass transport limitations,

There is no general or best method of immobilization; the protocol has to be developed individually for each catalyst.

The most common methods for the immobilization are entrapment in matrices such as alginate beads, cross-linking, and covalent or adsorptive binding to a carrier. A very recent method is the development of cross-linked enzyme crystals (CLECS) (see also Chapter 6)[23]. A survey of different immobilization methods can be found in in[24, 25].

19.3 Examples

The examples presented here are taken from[2]. Only those biotransformations were chosen where a classical chemical step was replaced. The enzymes involved are mainly from the groups of oxidoreductases (E.C. class 1) and hydrolases (E.C. class 3). There are a few examples of lyases (E.C. class 4) and one example of an isomerase (E.C. class 5). The processes involving oxidoreductases mainly use whole cells because of the problem of cofactor regeneration. The examples are sorted in the order of the main classes of the Enzyme Commission (E.C.). The big letter **E** denotes the biotransformation in the syntheses schemes.

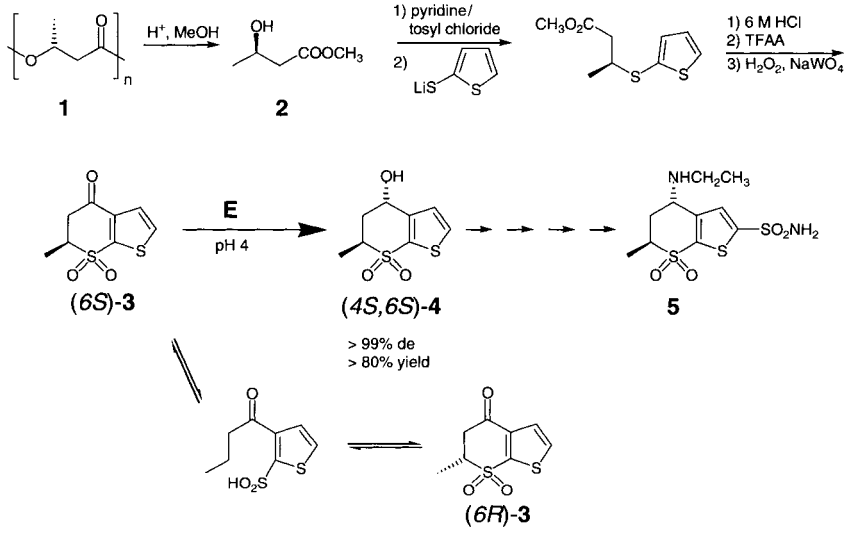
19.3.1
Reduction Reactions Catalyzed by Oxidoreductases (E.C. 1)

19.3.1.1
Ketone Reduction Using Whole Cells of *Neurospora crassa* (E.C. 1.1.1.1)[26–29]

The key step in the synthesis of Trusopt®, which is a topically active treatment for glaucoma, is the enantioselective reduction of 5,6-dihydro-6-methyl-4H-thieno[2,3b] thiopyran-4-one-7,7-dioxide (Fig. 19-1).

The biological route overcomes the problem of incomplete inversion of the *cis*-alcohol in the chemical synthesis (Fig. 19-2). The reaction is carried out below pH 5 to prevent epimerization of the (6*S*)-methyl ketosulfone in aqueous media.

The (*R*)-3-hydroxy-butyrate (Fig. 19-1), which is responsible for the stereochemistry of the methyl group in the sulfone ring, can be produced by depolymerization of biopolymers, e.g. Biopol from Zeneca. This is a natural polyester produced by some microorganisms as a storage compound.



1 = biologically derived homopolymer
2 = (*R*)-3-hydroxy-methyl butyrate
3 = 5,6-dihydro-6-methyl-4H-thieno[2,3b]thiopyran-4-one-7,7-dioxide
4 = 5,6-dihydro-4-hydroxy-6-methyl-4H-thieno[2,3b]thiopyran-7,7-dioxide
5 = trusopt, MK-0507
E = alcohol dehydrogenase, whole cells from *Neurosporia crassa*

Figure 19-1. Synthesis of Trusopt® (5) via enzymatic ketone reduction (Astra-Zeneca).

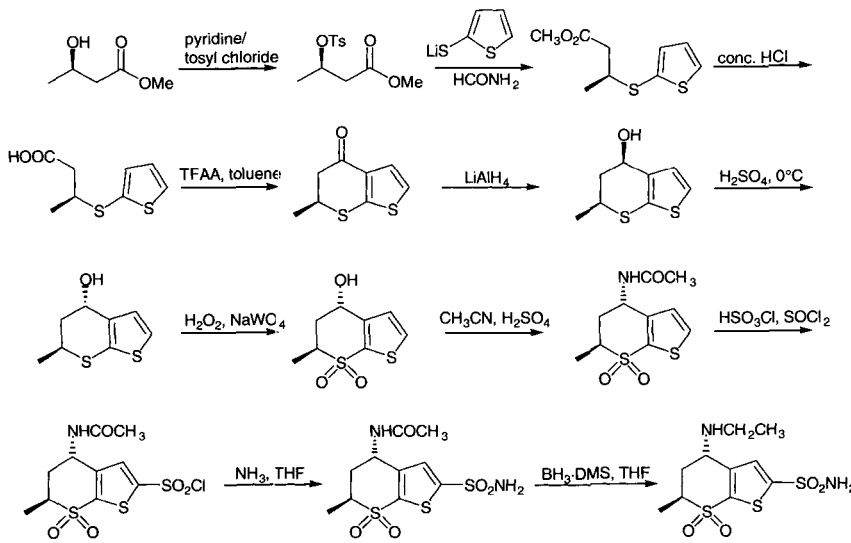
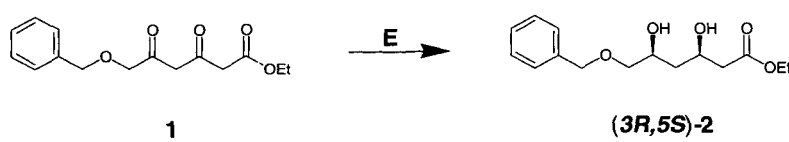


Figure 19-2. Chemical synthesis of Trusopt®.

19.3.1.2
Ketoester Reduction Using Cell Extract of *Acinetobacter calcoaceticus* (E.C. 1.1.1.1)[30–32]

The biotransformation (Fig. 19-3) is an alternative to the chemical synthesis via the chlorohydrine and selective hydrolysis of the acyloxy group (Fig. 19-4). After final fractional distillation this synthesis has an overall yield of 41%. The biotransformation has a yield of 92%. The diketoester can be obtained as shown in Fig. 19-5.

6-Benzyloxy-(*3R,5S*)-dihydroxy-hexanoic acid ethyl ester is a key chiral intermediate for anticholesterol drugs that act by inhibition of hydroxy methyl glutaryl coenzyme A (HMG CoA) reductase.



1 = 6-benzyloxy-3,5-dioxo-hexanoic acid ethyl ester
2 = 6-benzyloxy-3,5-dihydroxy-hexanoic acid ethyl ester
E = alcohol dehydrogenase from *Acinetobacter calcoaceticus*

Figure 19-3. Synthesis of key intermediate of anticholesterol drugs (Bristol-Myers Squibb).

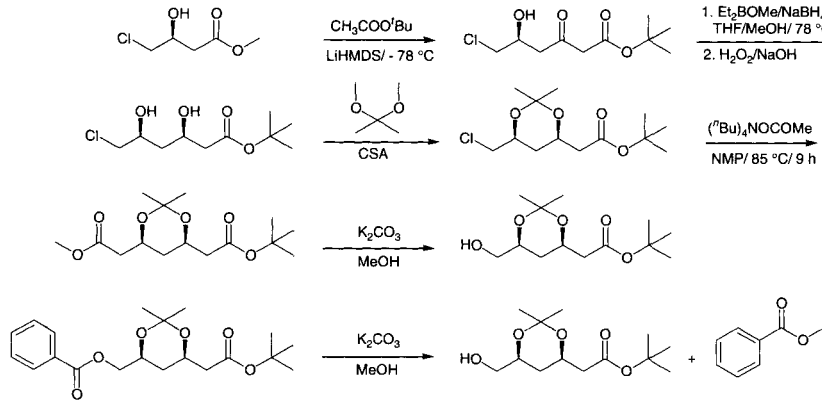


Figure 19-4. Chemical synthesis of key intermediate of anticholesterol drugs.

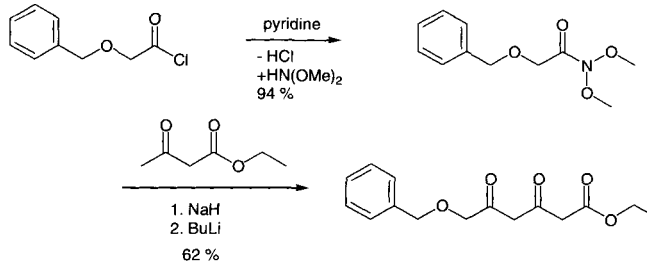
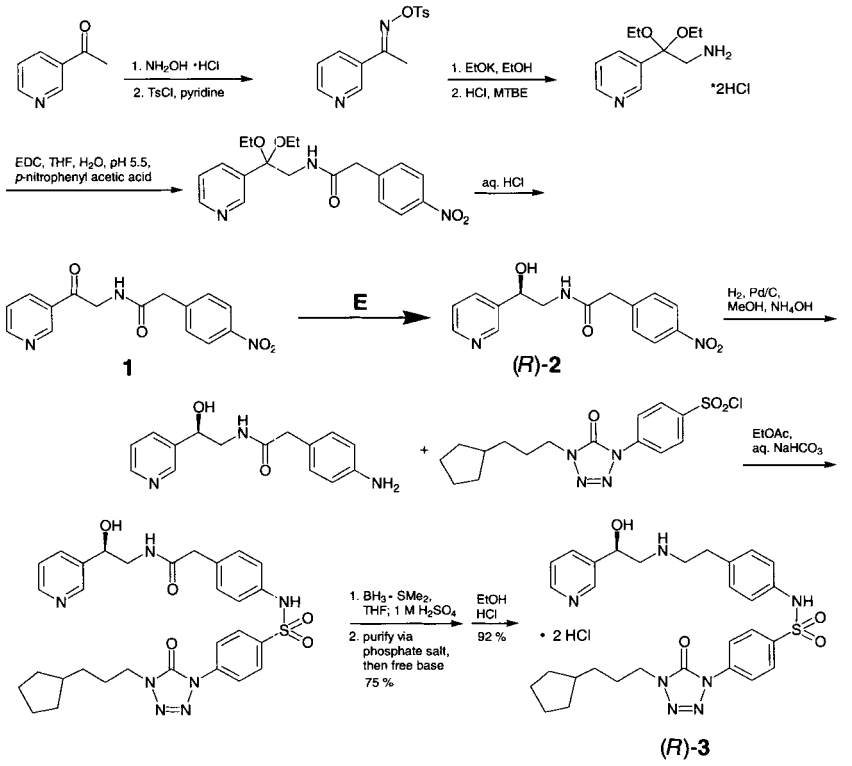


Figure 19-5. Synthesis of starting material 6-benzyloxy-3,5-dihydroxy-hexanoic acid ethyl ester.

19.3.1.3
Enantioselective Reduction with Whole Cells of *Candida sorbophila* (E.C. 1.1.X.X) [33–35]

Here the biotransformation (Fig. 19-6) is preferred over the chemical reduction with commercially available asymmetric catalysts (BH_3– or noble-metal-based), since with the chemocatalysts the desired high enantiomeric excess (*ee* > 98 %, 99.8 % after purification) is not achievable. Since the ketone has only a very low solubility in the aqueous phase, 1 kg ketone is added as solution in 4 L 0.9 M H_2SO_4 to the bioreactor. The bioreduction is essentially carried out in a two-phase system, consisting of the aqueous phase and small droplets made up of substrate and product. The downstream processing consists of multiple extraction steps with methyl ethyl ketone and precipitation induced by pH titration of the pyridine functional group (pK_a = 4.66) with NaOH. The (*R*)-amino alcohol is an important intermediate for the synthesis of β-3-agonists that can be used for obesity therapy and to decrease the level of associated type II diabetes, coronary artery disease and hypertension.



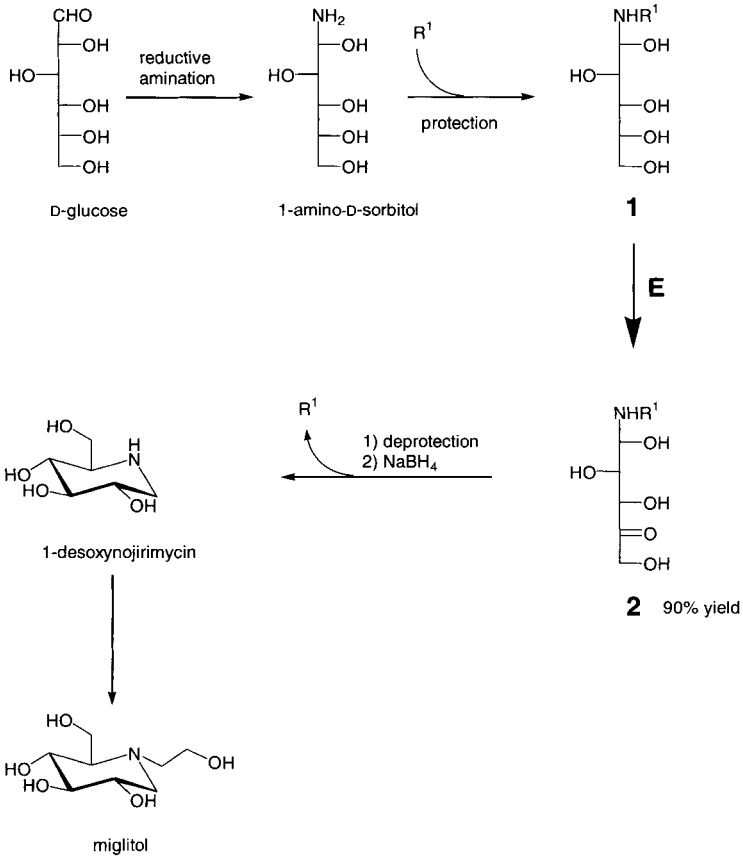
1 = 2-(4-nitro-phenyl)-*N*-(2-oxo-2-pyridin-3-ethyl)-acetamide
2 = (*R*)-*N*-(2-hydroxy-2-pyridin-3-yl-ethyl)-2-(4-nitro-phenyl)-acetamide
3 = β-3-agonist
E = dehydrogenase, whole cells of *Candida sorbophila*

Figure 19-6. Synthesis of key intermediate of β-3-agonist (Merck Research Laboratories).

19.3.2
Oxidation Reactions Catalyzed by Oxidoreductases (E.C. 1)

19.3.2.1
Alcohol Oxidation Using Whole Cells of *Gluconobacter suboxydans* (E.C. 1.1.99.21)[36–38]

In 1923 the bacterium *Acinetobacter suboxydans* was isolated and, starting in 1930, was used for the industrial oxidation of L-sorbitol to L-sorbose in the Reichstein-Grüssner synthesis of vitamin C[39]. Bayer uses the same type of reaction, but instead of *Acinetobacter* the bacterium *Gluconobacter suboxydans* is used in the oxidation of *N*-protected 6-amino-L-sorbitol to the corresponding 6-amino-L-sorbose, which is an intermediate in miglitol production (Fig. 19-7). 1-Desoxynojirimycin is produced by chemical intramolecular reductive amination of 6-amino-L-sorbose. In contrast, the



1 = 1-amino-D-sorbitol (N-protected)
2 = 6-amino-L-sorbose (N-protected)
E = D-sorbitol dehydrogenase, whole cells of *Gluconobacter oxidans*

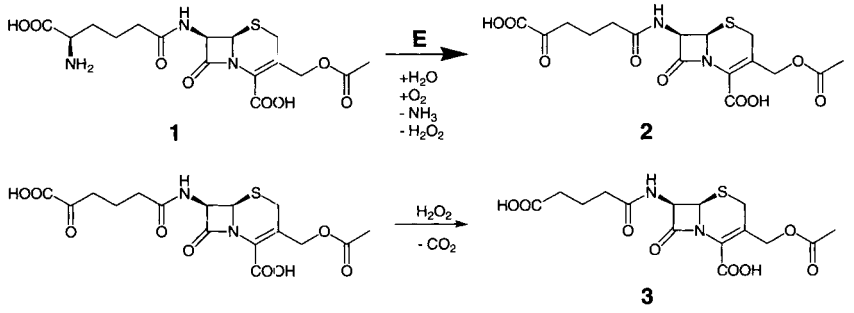
Figure 19-7. Synthesis of key intermediate for miglitol (Bayer).

published chemical synthesis of 1-desoxynojirimycin and its derivatives requires multiple steps and laborious protecting-group chemistry. Miglitol and derivatives thereof are pharmaceuticals for the treatment of carbohydrate metabolism disorders (e.g. diabetes mellitus).

19.3.2.2

Oxidative Deamination Catalyzed by Immobilized D-Amino Acid Oxidase from *Trigonopsis variabilis* (E.C. 1.4.3.3) [40–42]

This oxidative deamination catalyzed by immobilized enzymes is part of the 7-aminocephalosporanic acid (7-ACA) process. Ketoadipinyl-7-aminocephalosporanic acid decarboxylates *in situ* in the presence of H_2O_2, which is formed by the



1 = cephalosporin C
2 = α-ketoadipinyl-7-aminocephalosporanic acid
3 = glutaryl-7-aminocephalosporanic acid (7-ACA)
E = D-aminoacid oxidase, immobilized enzyme from *Trigonopsis variablis*

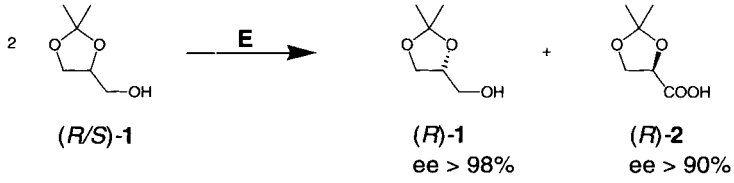
Figure 19-8. Synthesis of glutaryl-7-aminocephalosporanic acid (7-ACA) (Hoechst-Marion-Roussel).

biotransformation step yielding glutaryl-7-ACA. The reaction solution is directly transferred to the 7-ACA production (see Sect. 19.3.4.2 for details and a comparison with the chemical synthesis).

19.3.2.3

Kinetic Resolution by Oxidation of Primary Alcohols Catalyzed by Whole Cells from *Rhodococcus erythropolis* (E.C. 1.X.X.X) [43–45]

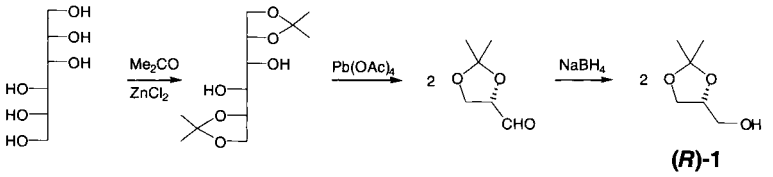
(*R*)-Isopropylideneglycerol is a useful C_3-synthon in the synthesis of (*S*)-β-blockers, e.g. (*S*)-metoprolol. Also, (*R*)-isopropylideneglyceric acid may be used as the starting material for the synthesis of biologically active products. The resolution is carried out by selective microbial oxidation of the (*S*)-enantiomer (Fig. 19-9). The chemical synthesis of (*R*)-isopropylideneglycerol starts either from unnatural L-mannitol or from L-ascorbic acid (Fig. 19-10). In comparison to the biotransformation, here stoichiometric quantities of lead tetra acetate are needed.



1 = isopropylideneglycerol
2 = isopropylideneglyceric acid
E = oxidase, whole cells from *Rhodococcus erythropolis*

Figure 19-9. Synthesis of (*R*)-isopropylideneglycerol and (*R*)-isopropylideneglyceric acid (International BioSynthetics).

From L-mannitol:



From L-ascorbic acid:

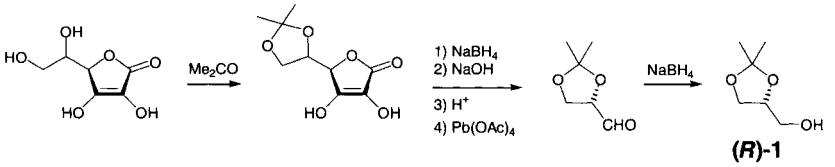
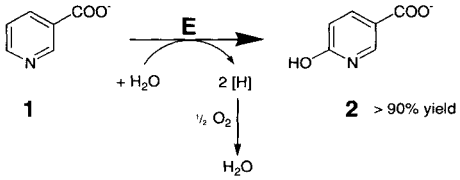


Figure 19-10. Chemical synthesis of (*R*)-isopropylideneglycerol.

19.3.2.4

Hydroxylation of Nicotinic Acid (Niacin) Catalyzed by Whole Cells of *Achromobacter xylosoxidans* (E.C. 1.5.1.13)[46–48]

6-Hydroxynicotinate is a versatile building block used chiefly in the synthesis of modern insecticides. The 6-hydroxynicotinate-producing strain (Fig. 19-11) was found by accident, when in the mother liquor of a niacin-producing chemical plant precipitated white crystals of 6-hydroxynicotinate were found. The second enzyme of the nicotinic acid pathway, the decarboxylating 6-hydroxynicotinate hydroxylase becomes strongly inhibited at niacin concentrations higher than 1%, whereas the operation of niacin hydroxylase is unaffected. In contrast to the biotransformation, the chemical synthesis of 6-substituted nicotinic acids is difficult and expensive because of the necessity for the separation of by-products that are produced by non-regioselective hydroxylations.



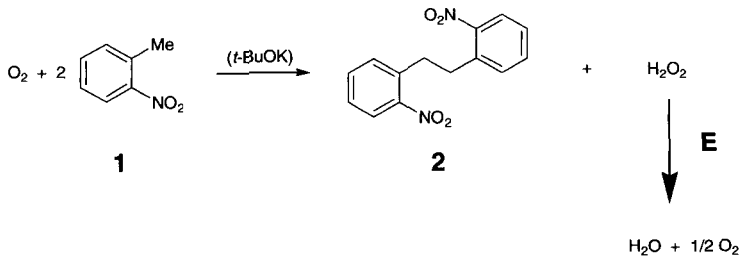
1 = niacin = nicotinic acid = pyridine-3-carboxylate
2 = 6-hydroxynicotinate = 6-hydroxy-pyridine-3-carboxylate
E = nicotinic acid hydrolase, whole cells from *Achromobacter xylosoxidans*

Figure 19-11. Synthesis of 6-hydroxynicotinate (Lonza).

19.3.2.5

Reduction of Hydrogen Peroxide Concentration by Catalase (E.C. 1.11.1.6)[49]

During oxidative coupling to dinitrodibenzyl (DNDB), hydrogen peroxide is formed as a by-product. It is not possible to decompose H_2O_2 by adding heavy-metal catalysts



1 = nitrotoluene
2 = dinitrodibenzyl (DNDB)
E = catalase, enzyme from microbial source

Figure 19-12. Degradation of hydrogen peroxide (Novartis).

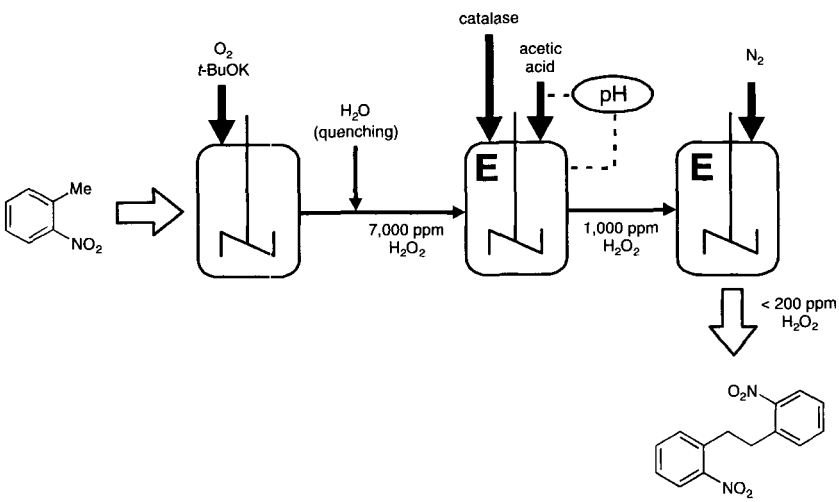


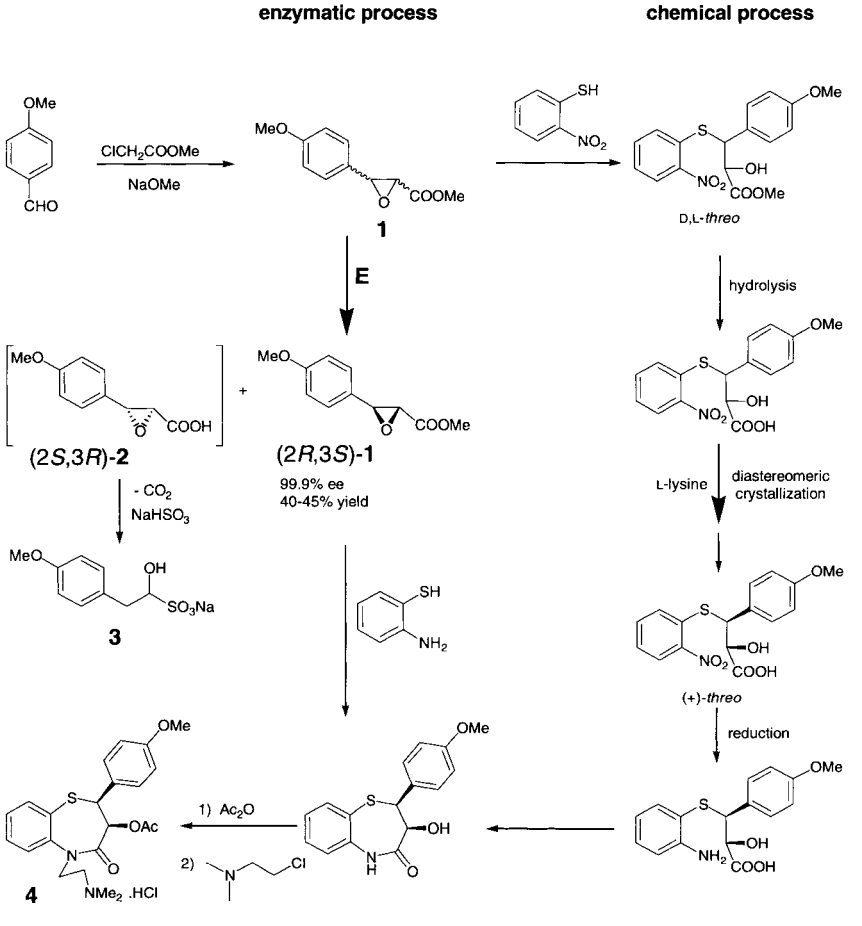
Figure 19-13. Flow scheme of dinitrodibenzyl synthesis.

because only an incomplete conversion is reached. Additionally, subsequent process steps with DNDB are problematic because of contamination with heavy-metal catalyst. The biotransformation is the only relevant method of decomposing the undesired side product H_2O_2 (Fig. 19-12). The enzyme of choice is catalase derived from a microbial source, which has advantages compared to beef catalase since the activity remains constant over a broad pH range from 6.0 to 9.0, temperatures up to 50 °C are tolerated, and salt concentrations up to 25% do not affect the enzyme stability. The reaction is carried out in a cascade of three continuously operated stirred tank reactors (Fig. 19-13). The H_2O_2 concentration is reduced from 7000 ppm to < 200 ppm in the product solution. The third vessel is aerated with nitrogen to degas the product solution. The dinitrodibenzyl is used as a pharmaceutical intermediate.

19.3.3
Hydrolytic Cleavage and Formation of C-O Bonds by Hydrolases (E.C. 3)

19.3.3.1
Kinetic Resolution of Glycidic Acid Methyl Ester by Lipase from *Serratia marcescens* (E.C. 3.1.1.3)[50–53]

Trans-(2R,3S)-(4-methoxyphenyl)glycidic acid methyl ester is an intermediate in the synthesis of diltiazem, a coronary vasodilator and a calcium channel blocker with



1 = *trans*-p-methoxyphenylmethylglycidate (MPGM)
2 = *trans*-p-methoxyphenylglycidic acid
3 = bisulfite adduct after decarboxylation
4 = diltiazem
E = lipase, enzyme from *Serratia marescens*

Figure 19-14. Comparison of chemical and biocatalytical route to diltiazem (Tanabe Seiyaku Co., Ltd.).

antianginal and antihypersensitive activity. It is produced worldwide in excess of > 100 t a^{-1}.

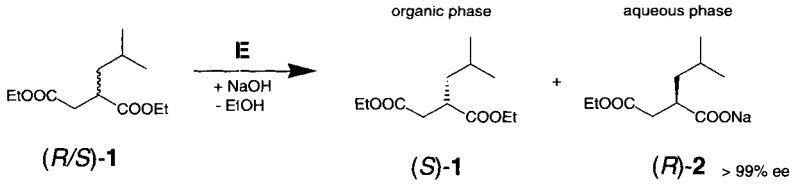
In comparison to the chemical route, only 5 steps (instead of 9) are necessary with the biotransformation (Fig. 19-14). The kinetic resolution is carried out in an earlier step with a lower molecular weight compound during the synthesis, resulting in a reduction of waste. By redesigning the synthesis route using a biotransformation, the manufacturing costs of diltiazem were decreased to two thirds of those of the original process including a chemical resolution [54].

The lipase from *Serratia marcescens* has a high enantioselectivity ($E = 135$) for the *(2R,3S)*-(4-methoxyphenyl)glycidic acid methyl ester, which acts as a competitive inhibitor. The formed acid (hydrolyzed (+)-methoxyphenylglycidate) is unstable and decarboxylates to give 4-methoxyphenylacetaldehyde; this aldehyde strongly inhibits and deactivates the enzyme. It is removed by transfer to the aqueous phase by formation of a water-soluble adduct with sodium hydrogen sulfite added to the aqueous phase. The bisulfite acts also as a buffer to maintain constant pH during synthesis.

The enantioselective hydrolysis is carried out in an organic-aqueous two-phase reactor (toluene/water), where the phase contact is established by a hydrophilic hollow-fiber membrane (polyacrylonitrile). The lipase is immobilized onto a spongy layer by pressurized adsorption. The productivity is about 40 kg *trans-(2R,3S)*-(4-methoxyphenyl)glycidic acid methyl ester m^{-2} a^{-1}. This process has been operated since 1993.

19.3.3.2
Kinetic Resolution of Diester by Protease Subtilisin Carlsberg from *Bacillus* sp. (E.C. 3.4.21.62) [55, 56]

(*R*)-(2-Methylpropyl)-butanedioic acid 4-ethyl ester is used as a chiral building block for potential collagenase inhibitors (e.g. Ro 31–9790) in the treatment of osteoarthritis. The diester is reacted as a 20% emulsion in 30 mM aqueous $NaHCO_3$ using Protease® L 660 or Alcalase® 2.5 L (9% each, with respect to the racemic diester) (Fig. 19-15). The unconverted (*S*)-diester can be extracted in a solvent such as toluene and racemized by heating the anhydrous extract with catalytic amounts of sodium ethanolate. The resulting racemic diester can be recycled, thus improving the overall



1 = (2-methylpropyl)butanedioic acid diethylether
2 = (2-methylpropyl)butanedioic acid 4-ethyl ester, Na-form
E = hydrolase, subtilisin Carlsberg from *Bacillus sp.*

Figure 19-15. Synthesis of (*R*)-(2-methylpropyl)butanedioic acid diethylether (Hoffmann La-Roche).

yield from 45% to 87%. The reaction was repeatedly carried out on a 200 kg scale with respect to the racemic diester. The enzyme is highly stereoselective even at high substrate concentrations (20%). The chemoenzymatic route starting from the cheap bulk agents maleic anhydride and isobutylene replaced the existing chemical research synthesis for bulk amounts (Fig. 19-16).

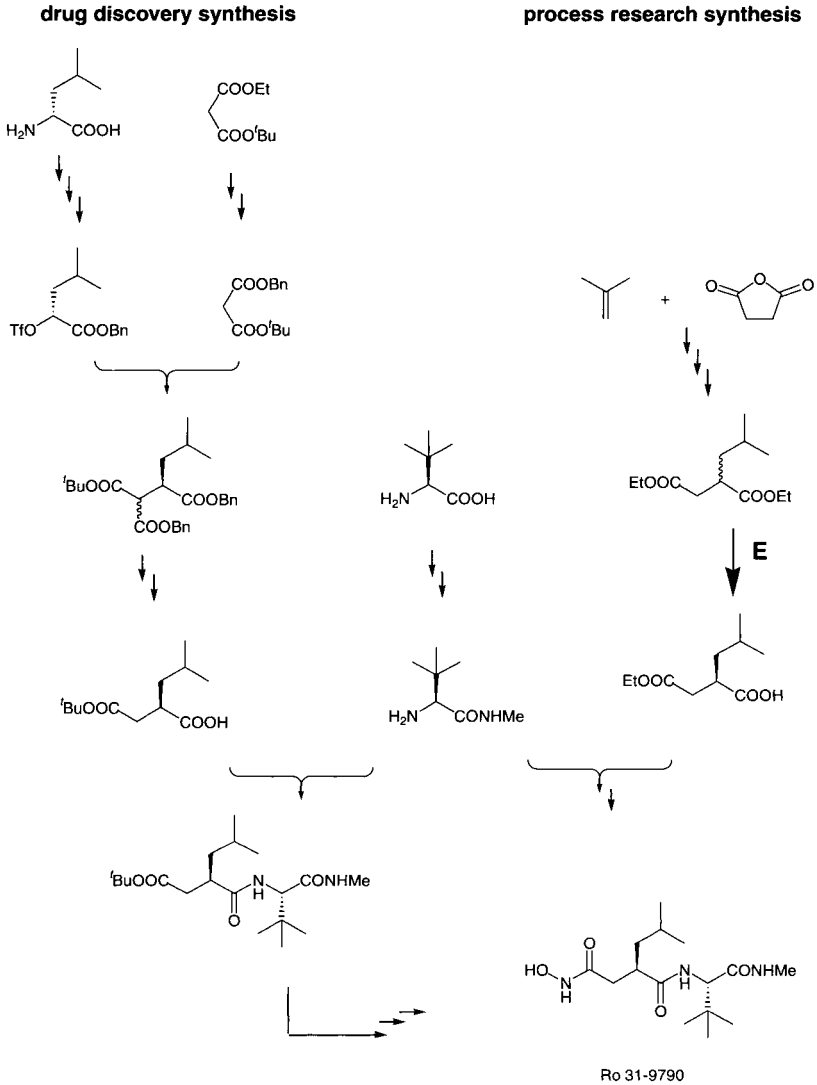
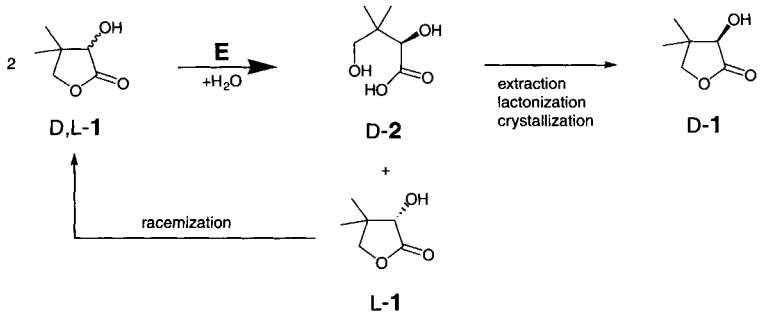


Figure 19-16. Comparison of drug discovery and process research route of Ro 31-9790.



1 = pantolactone
2 = pantoic acid
E = lactonase, whole cells from *Fusarium oxysporum*

Figure 19-17. Synthesis of D-pantolactone (Fuji Chemical Industries).

19.3.3.3

Kinetic Resolution of Pantolactones and Derivatives thereof by a Lactonase from *Fusarium oxysporum* (E.C. 3.1.1.25)[57]

Pantenoic acid is used as a vitamine B_2 complex. D- and L-pantolactone are used as chiral intermediates in chemical synthesis. The enantioselective hydrolysis is carried out in the aqueous phase with a substrate concentration of 2.69 M = 350 g L^{-1} (Fig. 19-17). For the synthesis whole cells are immobilized in calcium alginate beads and used in a fixed bed reactor. The immobilized cells retain more than 90% of their initial activity after 180 days of continuous use. At the end of the reaction L-pantolactone is extracted and reracemized to D,L-pantolactone, which is recycled to the reactor. The D-pantenoic acid is chemically lactonized to D-pantolactone and extracted. By applying cells from *Brevibacterium protophormia* the L-lactone is available. The biotransformation eliminates several steps that are necessary in the chemical resolution process (Fig. 19-18).

19.3.3.4

Hydrolysis of Starch to Glucose by Action of Two Enzymes: α-Amylase (E.C. 3.2.1.1) and Amyloglucosidase (E.C. 3.2.1.3)[58–60]

The process is part of the production of high fructose corn syrup. After several improvements, this process (Fig. 19-19) provides an effective way for an important, low-cost sugar substitute derived from grain. At various stages enzymes are applied in this process[61, 62]. The corn kernels are softened to separate oil, fiber and proteins by centrifugation. The enzymatic steps are cascaded to yield the source product for the invertase process after liquefaction in continuous cookers, debranching and filtration (Fig. 19-20). Since starches from different natural sources have different compositions, the procedure is not unique. The process ends, if all starch is completely broken down to limit the amount of oligomers of glucose and dextrins. Additionally, recombination of molecules has to be prevented. The thermostable

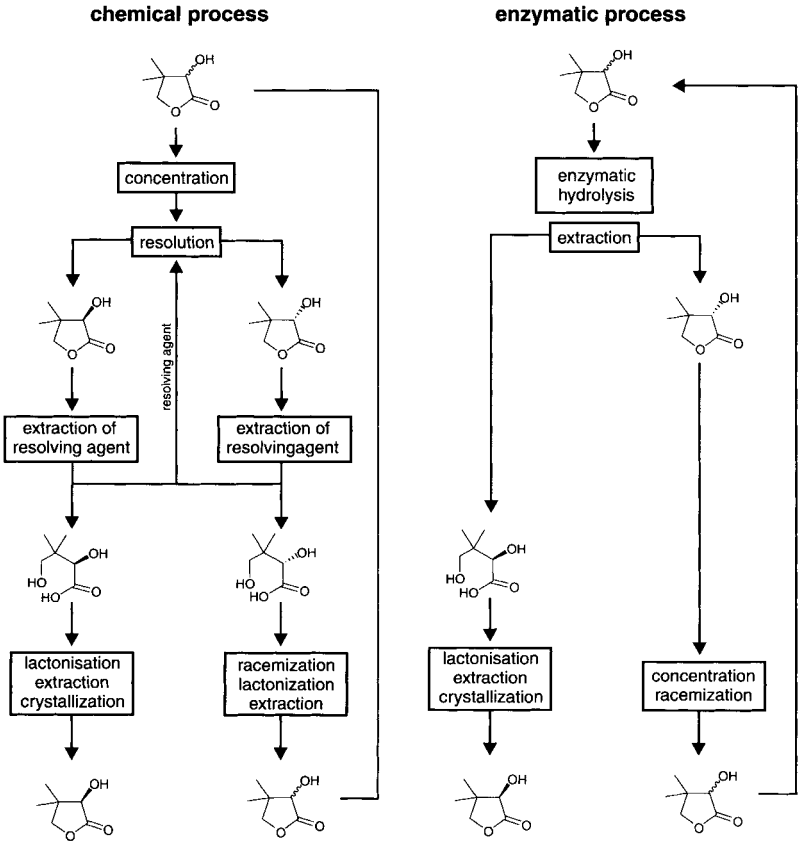
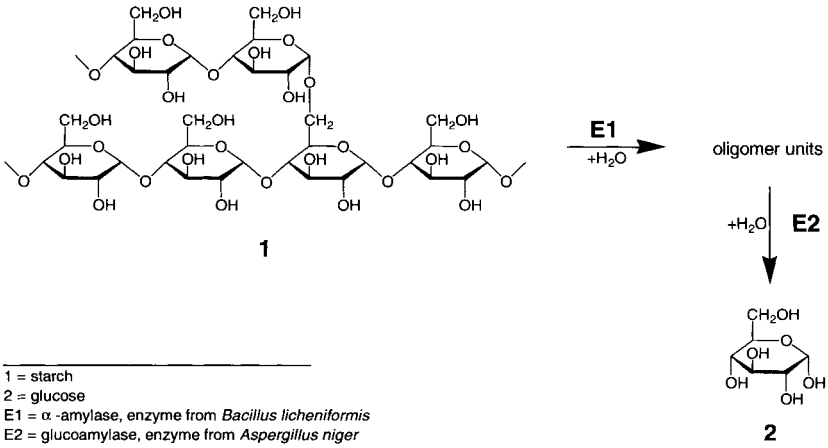


Figure 19-18. Comparison of chemical and biocatalytic route for the enantioselective synthesis of pantolactone.



1 = starch
2 = glucose
E1 = α -amylase, enzyme from *Bacillus licheniformis*
E2 = glucoamylase, enzyme from *Aspergillus niger*

Figure 19-19. Synthesis of glucose (Several companies).

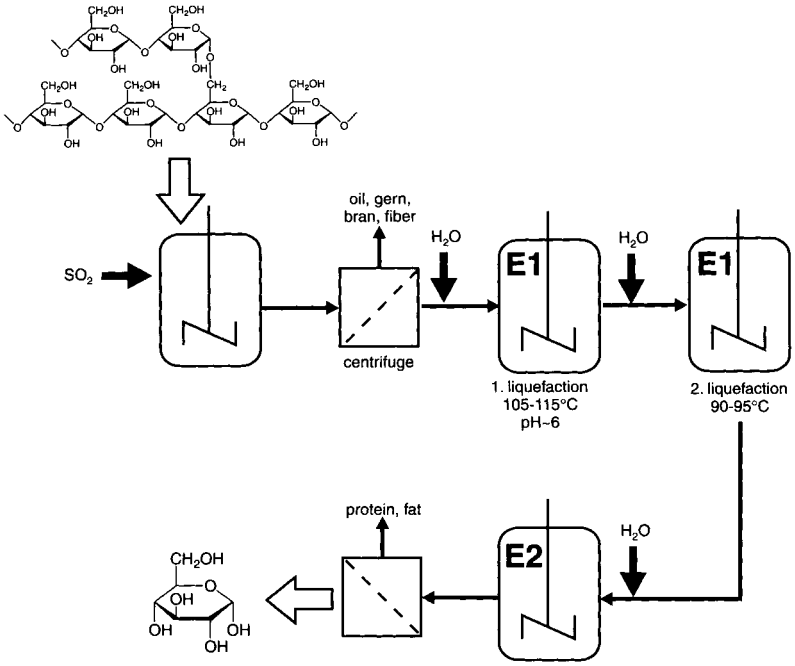


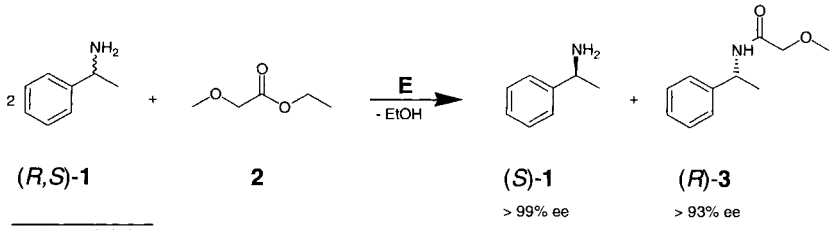
Figure 19-20. Flow scheme for the hydrolysis of starch to glucose.

enzyme can be used up to 115 °C. The enzymes need Ca^{2+} ions for stabilization and activation. Since several substances in corn can complex cations, the cation concentration is increased requiring a further product purification, i.e. making it necessary to refine the product. There is no alternative industrial chemical process for starch liquefaction. The worldwide production is about 10^7 t a^{-1}.

19.3.4
Formation or Hydrolytic Cleavage of C-N Bonds by Hydrolases (E.C. 3)

19.3.4.1
Enantioselective Acylation of Racemic Amines Catalyzed by Lipase from *Burkholderia plantarii* (E.C. 3.1.1.3)[63–65]

The lipase catalyzes the kinetic resolution of racemic amines, e.g. 1-phenylethylamine (Fig. 19-21)[11]. Products are intermediates for pharmaceuticals and pesticides. They can also be used as chiral synthons in asymmetric synthesis. As acylating agent ethylmethoxyacetate is used, because the reaction rate is more than 100 times faster than that with butyl acetate. Probably an enhanced carbonyl activity induced by the electronegative α-substituents accounts for the activating effect of the methoxy group. The lipase is immobilized on polyacrylate. The lowered activity caused by use of in organic solvent (*tert*-methylbutylether = MTBE) can be increased



1 = 1-phenylethylamine
2 = ethylmethoxyacetate
3 = phenylethylmethoxyamide
E = lipase, enzyme from *Burkholderia plantarii*

Figure 19-21. Kinetic resolution of phenylethylamine (BASF).

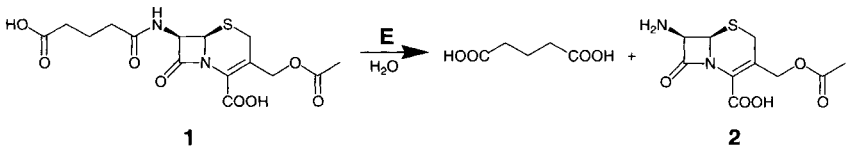
(about 1000 times and more) by freeze drying a solution of the lipase together with fatty acids (e.g. oleic acid). Because of the use of MTBE a high starting material concentration of 1.65 M 1-phenylethylamine can be established. The enantioselectivity is greater than 500. The (*R*)-phenylethylmethoxyamide can easily be hydrolyzed to the (*R*)-phenylethylamine. The unconverted (*S*)-enantiomer can be racemized using a palladium catalyst.

19.3.4.2

7-Aminocephalosporanic Acid Formation by Amide Hydrolysis Catalyzed by Glutaryl Amidase (E.C. 3.1.1.41) [66–69]

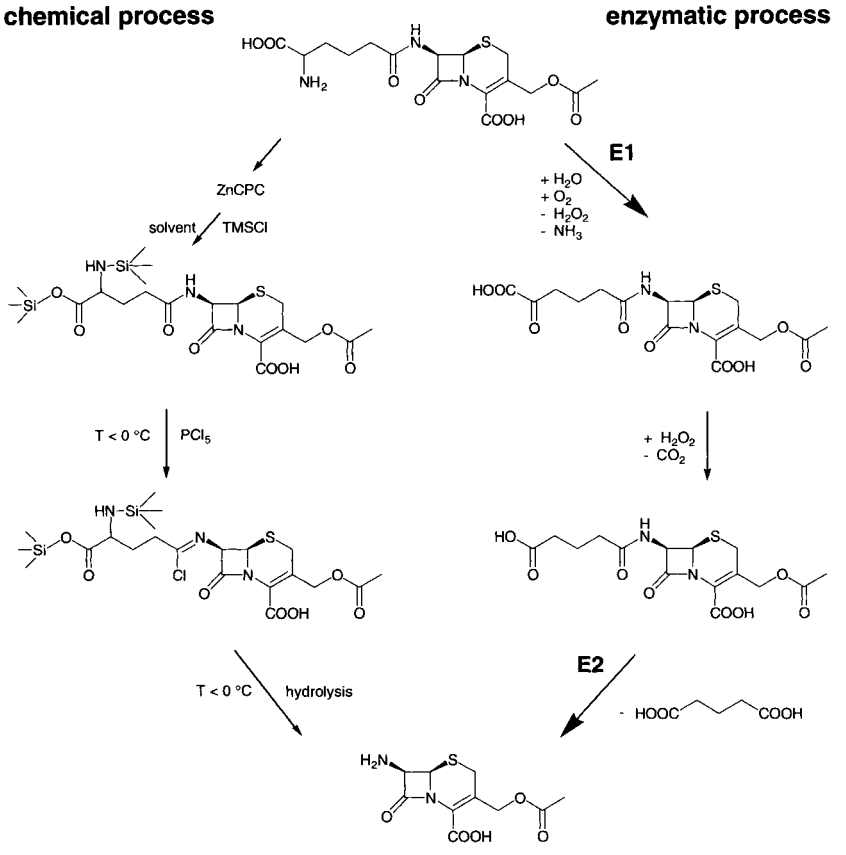
The second step of the 7-aminocephalosporanic acid (7-ACA) process is the deamidation of glutaryl-7-ACA (Fig. 19-22), the first step is described in Sect. 19.3.2.2. 7-ACA is an intermediate for semi-synthetic cephalosporins. Hoechst Marion Roussel uses the glutaryl amidase immobilized on a spherical carrier. Toyo Jozo and Asahi Chemical immobilize the glutaryl amidase on porous styrene anion exchange resin with subsequent cross-linking with 1% glutardialdehyde. The catalyst is applied in a fixed bed reactor in a repetitive batch mode (70 cycles). Here, an enzymatic process has replaced an existing chemical process for environmental reasons (Fig. 19-23):

In the first step, the zinc salt of cephalosporin C is produced, followed by the protection of the functional groups (NH_2 and COOH) with trimethylchlorosilane.



1 = glutaryl-7-aminocephalosporanic acid
2 = 7-aminocephalosporanic acid (7-ACA)
E = glutaryl amidase, enzyme from *Escherichia coli*

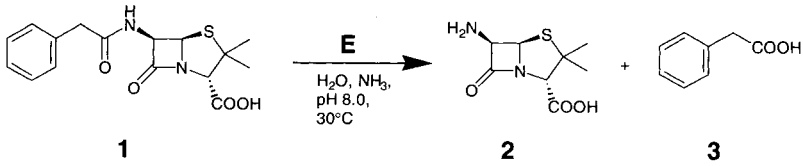
Figure 19-22. Synthesis of 7-aminocephalosporanic acid (7-ACA) (Asahi Chemical, Hoechst Marion Roussel, Toyo Jozo).



E1 = D-aminoacid oxidase
E2 = glutaryl amidase

Figure 19-23. Comparison of chemical and biocatalytical route for the synthesis of 7-ACA.

The imide chloride is synthesized in the subsequent step at 0 °C with phosphorous pentachloride. Hydrolysis of this imide chloride yields 7-ACA. By replacement of this synthesis with the biotransformation, the use of heavy-metal salts ($ZnCl_2$) and chlorinated hydrocarbons as well as precautions for highly flammable compounds can be circumvented. The off-gas quantities were reduced from 7.5 to 1.0 kg. Mother liquors requiring incineration were reduced from 29 to 0.3 t. Residual zinc that was recovered as $Zn(NH_4)PO_4$ is reduced from 1.8 to 0 t. The absolute costs of environmental protection are reduced by 90% per tonne of 7-ACA. Asahi Chemical and Toyo Jozo have produced 7-ACA since 1973 with a capacity of 90 t a^{-1} and Hoechst Marion Roussel since 1996 with a capacity of 200 t a^{-1}.



1 = penicillin-G
2 = 6-amino penicillanic acid (6-APA)
3 = phenylacetic acid
E = penicillin amidase, enzyme from *Escherichia coli*

Figure 19-24. Synthesis of 6-amino penicillanic acid (multiple companies).

19.3.4.3

Penicillin G Hydrolysis by Penicillin Amidase from *Escherichia coli* (E.C. 3.5.1.11)[68–71]

6-Amino penicillanic acid (6-APA) is used as the intermediate for manufacturing semi-synthetic penicillins. Companies applying this technology (Fig. 19-24) include Unifar, Turkey; Asahi Chemicals, Japan; Fujisawa Pharmaceutical Co., Japan; Gist-Brocades/DSM, The Netherlands; Novo-Nordisk, Sweden; Pfizer, USA. The enzyme is isolated and immobilized, often on Eupergit®C (Röhm, Germany). The production is carried out in a repetitive batch mode. The immobilized enzyme is retained by sieves. In case of the Eupergit®C immobilized amidase the residual activity is about 50% of initial activity after 800 batch cycles. Therefore the hydrolysis time after 800 batch cycles increases from initially 60 min to 120 min. The space-time yield is 445 g L^{-1} d^{-1}. Phenylacetic acid is removed by extraction and 6-APA can be crystallized. Concentrating the "split" solution and/or the mother liquor of crystallization via vacuum evaporation or reverse osmosis can increase the yield. The production plant operates for 300 days per year with an average production of 12.8 batch cycles per day (production campaigns of 800 cycles per campaign). Asahi Chemical utilizes a penicillin amidase from *Bacillus megaterium* that is immobilized on aminated porous polyacrylonitrile fibers. The production is carried out in a recirculation reactor consisting of 18 parallel columns with immobilized enzyme. Each column has a volume of 30 L. The circulation of the reaction solution is established with a flow rate of 6 000 L h^{-1}. One cycle time takes 3 h. The lifetime of each column is 360 cycles. Purification of 6-APA is done by isoelectric precipitation at pH 4.2 with subsequent filtration and washing with methanol.

7-Amino deacetoxy cephalosporanic acid (7-ADCA) is also produced by the same technology.

Several chemical steps are replaced by a single enzyme reaction (Fig. 19-25). Organic solvents, the use of low temperature (– 40 °C) and the need for absolutely anhydrous conditions, which used to make the process difficult and expensive, are no longer necessary in the enzymatic process.

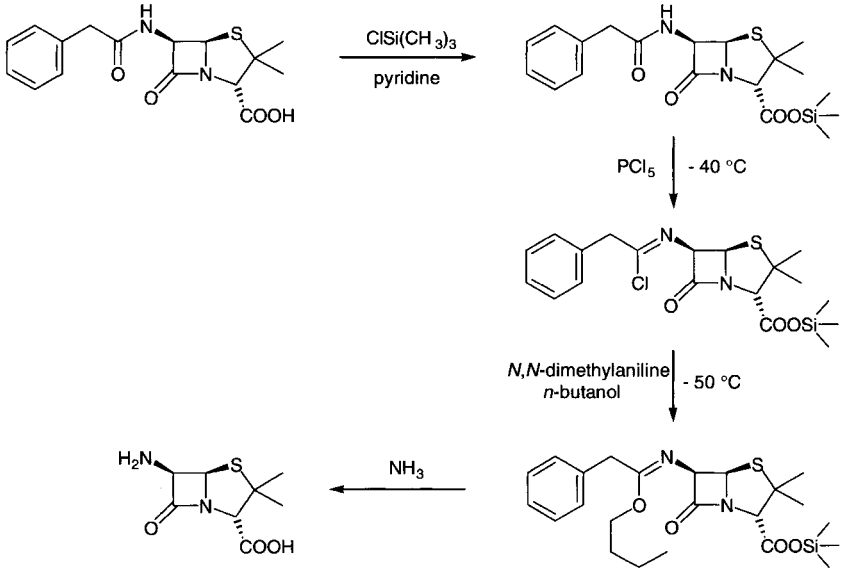


Figure 19-25. Chemical process for 6-APA.

19.3.4.4

Kinetic Resolution of α-Amino Acid Amides Catalyzed by Aminopeptidase from *Pseudomonas putida* (E.C. 3.4.1.11)[72–75]

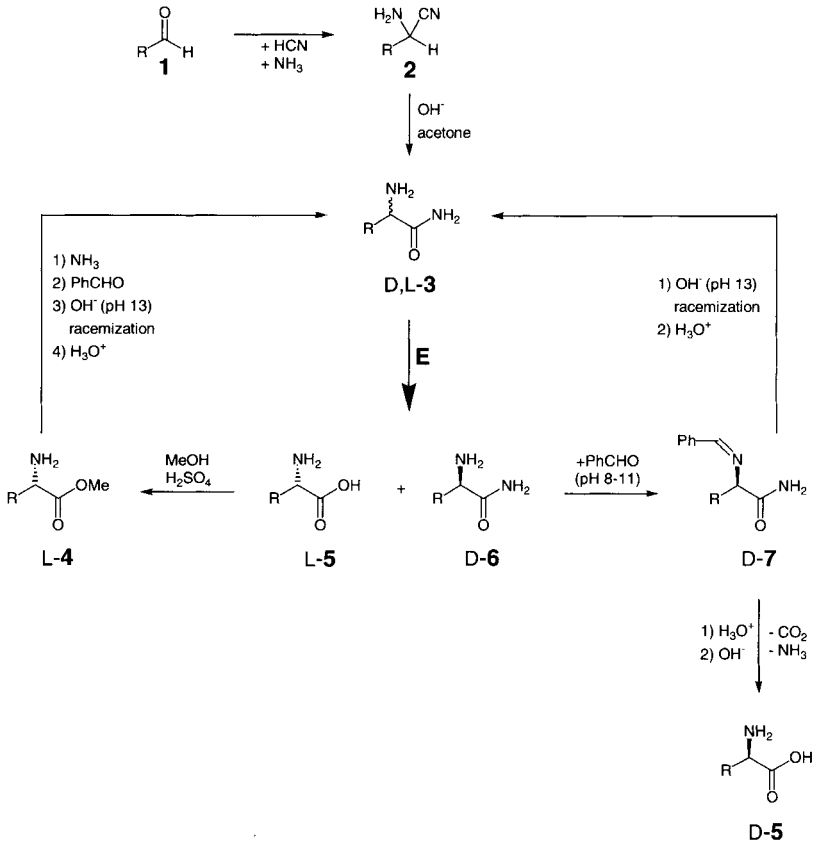
Enantiomerically pure α-H-amino acids are intermediates in the synthesis of antibiotics used for parenteral nutrition and for food and feed additives (see also Chapter 12.2). Examples are D-phenylglycine and 4-hydroxyphenylalanine for semi-synthetic β-lactam antibiotics and L-phenylalanine for the peptidic sweetener aspartame. DSM used this process to produce also L-homophenylalanine, a potential precursor molecule for several ACE-inhibitors.

The α-amino amides as substrates for this enantiospecific, biocatalytic amide hydrolysis can be readily obtained from the appropriate aldehydes via the Strecker synthesis (Fig. 19-26).

As whole cell catalyst, *Pseudomonas putida*, which accepts a wide range of substrates, is applied. Subsequent to the biotransformation, benzaldehyde is added, resulting in precipitation of the D-amide Schiff base, which can be easily isolated by filtration. An acidification step leads to the D-amino acid. The L-amino acid can be reused after racemization so that a theoretical yield of 100% D-amino acid is possible.

The same process can be used for the synthesis of 100% of L-amino acids by racemizing the Schiff base of the D-amide in a short time using small amounts of base in organic solvents.

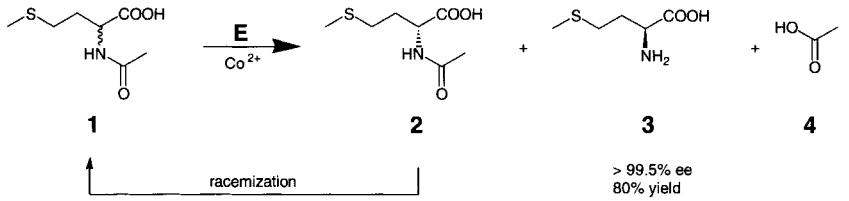
Using *in vivo* protein engineering not only mutant strains of *Pseudomonas putida*



1 = aldehyde
2 = amino nitrile
3 = α-amino acid amide
4 = α-amino acid methyl ester
5 = α-amino acid
6 = α-amino acid amide
7 = schiff base of α-amino acid amide
E = aminopeptidase, whole cells from *Pseudomonas putida*

Figure 19-26. Production of L-and D-α-amino acids by kinetic resolution of α-amino acids amides (DSM).

exhibiting L-amidase and also D-amidase but also amino acid amide racemase activities were obtained. Using these mutants a convenient synthesis of α-H-amino acids with 100% yield would be possible with one cell system. It is noteworthy that only α-H-substrates can be used. By screening, a new biocatalyst of the strain *Mycobacterium neoaurum* was found, which is capable of converting α-substituted amino acid amides.



1 = *N*-acetyl-D,L-methionine
2 = *N*-acetyl-D-methionine
3 = L-methionine
4 = acetic acid
E = aminoacylase, enzyme from *Aspergillus niger*

Figure 19-27. Biocatalytical production of L-methionine by kinetic resolution (Degussa).

19.3.4.5

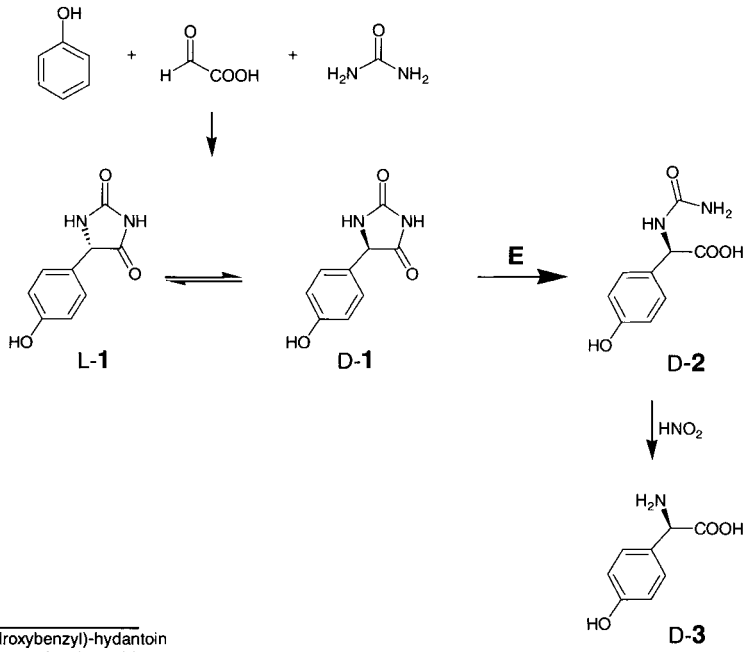
Production of L-Methionine by Kinetic Resolution with Aminoacylase of *Aspergillus oryzae* (E.C. 3.5.1.14) [76–79]

The *N*-acetyl-D,L-amino acid precursors are conveniently accessible through either acetylation of D,L-amino acids with acetyl chloride or acetic anhydride in a Schotten-Baumann reaction or via amidocarbonylation [80]. For the acylase reaction, Co^{2+} as metal effector is added to yield an increased operational stability of the enzyme. The unconverted acetyl-D-methionine is racemized by acetic anhydride in alkali, and the racemic acetyl-D,L-methionine is reused. The racemization can also be carried out in a molten bath or by an acetyl amino acid racemase. Product recovery of L-methionine is achieved by crystallization, because L-methionine is much less soluble than the acetyl substrate. The production is carried out in a continuously operated stirred tank reactor. A polyamide ultrafiltration membrane with a cutoff of 10 kDa retains the enzyme, thus decoupling the residence times of catalyst and reactants. L-methionine is produced with an *ee* > 99.5% and a yield of 80% with a capacity of > 300 t a^{-1}. At Degussa, several proteinogenic and non-proteinogenic amino acids are produced in the same way e.g. L-alanine, L-phenylalanine, α-amino butyric acid, L-valine, L-norvaline and L-homophenylalanine.

19.3.4.6

Production of D-*p*-Hydroxyphenyl Glycine by Dynamic Resolution with Hydantoinase from *Bacillus brevis* (E.C. 3.5.2.2) [8, 81–83]

D-*p*-Hydroxyphenyl glycine is a key raw material for the semisynthetic penicillins such as ampicillin and amoxycillin. It is also used in photographic developers. Racemic hydantoins are synthesized starting from phenol derivatives, glyoxylic acid and urea via the Mannich condensation (Fig. 19-28). The D-specific hydantoinase is applied as immobilized whole cells in a batch reactor. The unreacted L-hydantoins are readily racemized under the alkaline conditions (pH 8) of enzymatic hydrolysis, yielding quantitative conversion. This process enables the stereospecific preparation of various amino acids, such as L-tryptophane, L-phenylalanine, D-valine, D-alanine



1 = 5-(p-hydroxybenzyl)-hydantoin
2 = D-*N*-carbamoyl amino acid
3 = D-4-hydroxyphenyl glycine
E = D-hydantoinase, whole cells from *Bacillus brevis*

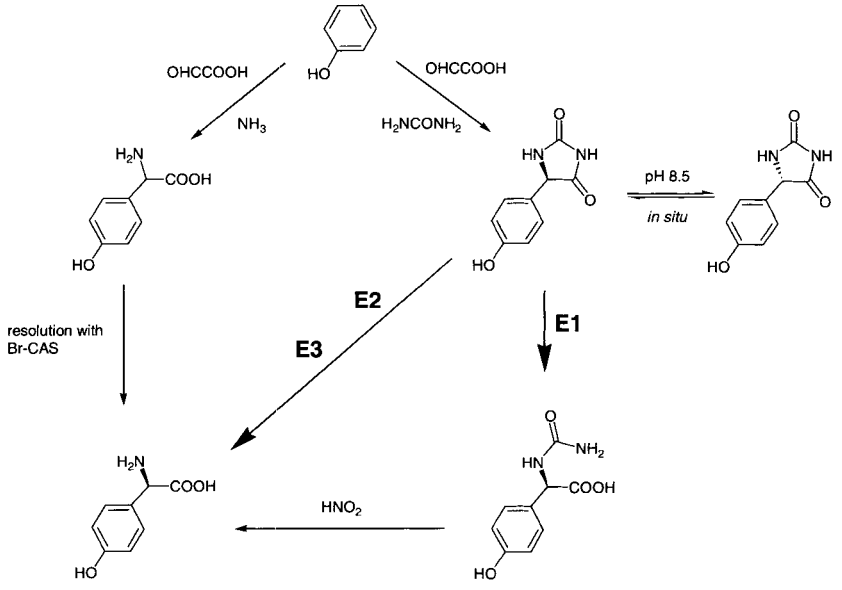
Figure 19-28. Synthesis of D-amino acids (Kanegafuchi).

and D-methionine. Instead of chemical treatment with sodium nitrite, a carbamoylase (EC 3.5.1.77) can also be applied to remove the carbamoyl group. Several other companies have developed patented processes to produce D-hydroxyphenylglycine (Ajinomoto, DSM, SNAM-Progetti, Recordati and others).

Here the biotransformation competes with the classical chemical route (Fig. 19-29), which employs bromocamphorsulfonic acid (Br-CAS) as the resolving agent. In both routes phenol is used as raw material since *p*-hydroxybenzaldehyde is too expensive. The hydantoinase process for phenylglycines does not necessarily need an extra racemization step since the hydantoin is racemized *in situ* at an alkaline pH. Because of the dynamic resolution in the case of this biotransformation, higher yields are reached.

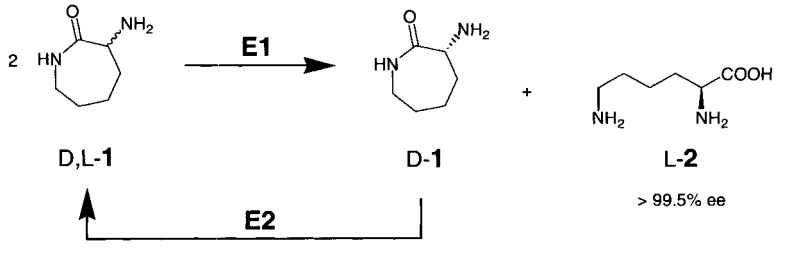
19.3.4.7
Dynamic Resolution of α-Amino-ε-caprolactam by the Action of Lactamase (E.C. 3.5.2.11) and Racemase (E.C. 5.1.1.15)[84, 85]

Again a dynamic resolution is carried out, but this time the racemization is introduced by an enzyme, a racemase from *Achromobacter obae* (Fig. 19-30). The lactamase and racemase are applied as whole cells and are fortunately active at the same pH, so that they can be used in one reactor. Reaction conditions enabling



E1 = D-hydantoinase, whole cells from *Bacillus brevis*
E2/E3 = D-hydantoinase/*N*-carbamoyl-D-amino acid hydrolase, whole cells, strain *Pseudomonas* sp. contains both enzymes

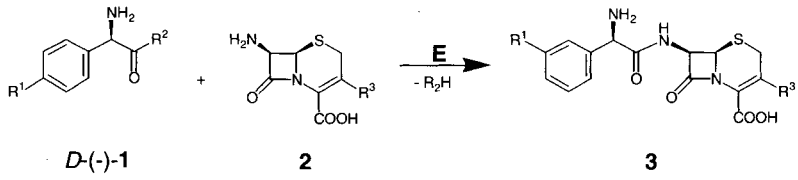
Figure 19-29. Comparison of chemical and biocatalytical route for the synthesis of D-amino acids (Kanegafuchi).



1 = α-amino-ε-caprolactam (ACL)
2 = lysine
E1= L-aminolactam-hydrolase, whole cells from *Cryptococcus laurentii*
E2= amino-lactam-racemase, whole cells from *Achromobacter obae*

Figure 19-30. Synthesis of L-lysine (Toray Industries).

chemical racemization would reduce the enzyme stability. L-Lysine was produced with an *ee* of 99.5 % at a capacity of 4 000 t a^{-1}. This process has been totally replaced by highly effective fermentation methods.



1a = phenylglycineamide (R^1=H, R^2=NH_2) = PGA
1b = phenylglycinemethylester (R^1=H, R^2=OMe) = PGM
1c = hydroxyphenylglycineamide (R^1=OH, R^2=NH_2) = HPGA
1d = hydroxyphenylglycinemethylester (R^1=OH, R^2=OMe) = HPGM
2a = 7-aminodeacetoxycephalosporanic acid (R^3=Me) = 7-ADCA
2b = 7-aminodeacetoxymethyl-3-chlorocephalosporanic acid (R^3=Cl) = 7-ACCA
3a = cefaclor (R^1=H, R^3=Cl)
3b = cephalexin (R^1=H, R^3=Me)
3c = cefadroxil (R^1=OH, R^3=Me)
E = penicillin acylase

Figure 19-31. Synthesis of β-lactam antibiotics (Chemferm).

19.3.4.8
Synthesis of β-Lactam Antibiotics Catalyzed by Penicillin Acylase (E.C. 3.5.1.11)[86–89]

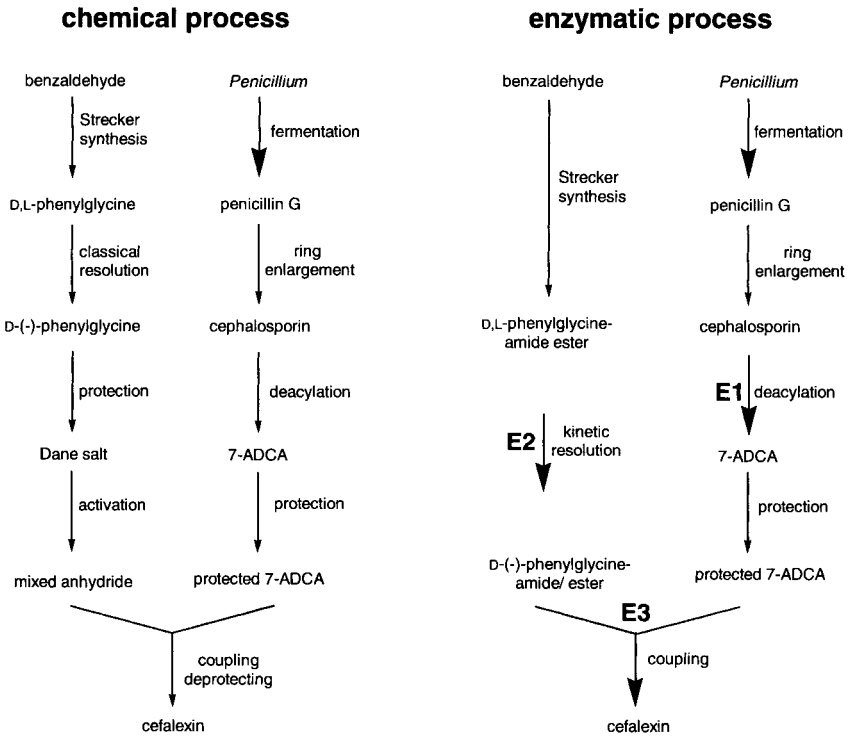
The penicillin acylases do not accept charged amino groups. Therefore phenylglycine itself cannot be used at a pH value at which the carboxyl function is uncharged, because the amino group will then be charged.

To reach non-equilibrium concentrations of the product, the substrate must be activated as an ester or amide (Fig. 19-31). By this means the amino group can be partly uncharged at the optimal pH value of the enzyme. In biological systems, ATP delivers the activation energy. Using the same synthetic pathway alternatively to 7-ADCA and 7-ACCA, 6-APA derivatives can also be synthesized.

The established chemical synthesis started from benzaldehyde and included the fermentation of penicillin (Fig. 19-32). The process consists of ten steps with a waste stream of 30–40 kg waste per kg product. The waste contains methylene chloride, other solvents, silylating agents and many products from side-chain protection and acylating promoters. In comparison, the chemoenzymatic route needs only six steps including three biocatalytic ones. The biotransformations E1 and E2 in Fig. 19-32 can be found in Sect. 19.3.4.3 and 19.3.4.4.

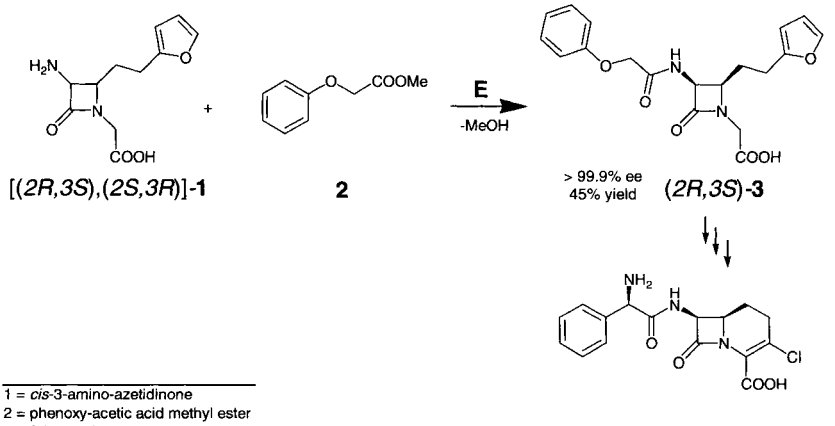
19.3.4.9
Synthesis of Azetidinone β-Lactam Derivatives Catalyzed by Penicillin Acylase (E.C. 3.5.1.11)[90, 91]

It was thought that the Pen G amidase would exhibit only a limited substrate spectrum, since it does not hydrolyze the phenoxyacetyl side chain of penicillin V. Nevertheless, Eli Lilly shows that the Pen G amidase acylates the amino function of *cis*-3-amino-azetidinone with the methyl ester of phenoxyacetic acid (Fig. 19-33). The



E1 = penicillin amidase
E2 = amino peptidase
E3 = penicillin acylase

Figure 19-32. Comparison of the chemical and biocatalytical synthesis of cefalexin.



1 = *cis*-3-amino-azetidinone
2 = phenoxy-acetic acid methyl ester
3 = β-lactam intermediate
4 = loracarbef
E = Pen G amidase, enzyme from *Escherichia coli*

Figure 19-33. Synthesis of azetidione β-lactam derivatives (Eli Lilly).

acylation occurs using methyl phenylacetate (MPA) or methyl phenoxyacetate (MPOA) as the acylating agents. The penicillin amidase is immobilized on Eupergit (Roehm GmbH, Germany).

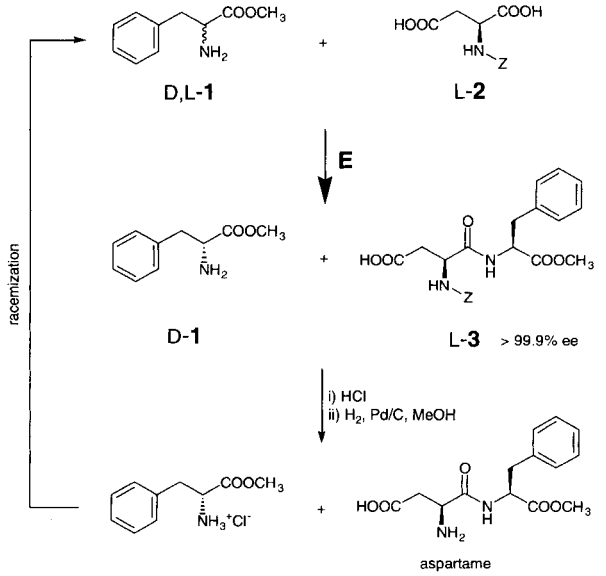
The chemical resolution of the racemic azetidinone is only low yielding. The (2*R*,3*S*)-azetidinone is a key intermediate in the synthesis of the carbacephalosporin antibiotic loracarbef.

19.3.4.10
Enantioselective Synthesis of an Aspartame Precursor with Thermolysin from *Bacillus proteolicus* (E.C. 3.4.24.27) [92, 93]

Since the reaction (Fig. 19-34) is limited by the equilibrium the products have to be removed from the reaction mixture to reach high yields. Therefore an excess of racemic phenylalanine methylester (which is inert to the reaction) is added. The carboxylic anion of the protected aspartame forms a poorly soluble adduct with D-Phe-OCH_3 that precipitates from the reaction mixture. The precipitate can be removed easily by filtration. Final steps of the process are the separation of D-Phe-ester, removal of protecting groups and racemization of the formed L-amino acid. α-Aspartame is produced with > 99.9% and a worldwide capacity of ~ 10,000 t a^{-1}, ~ 2,500 t a^{-1} by enzymatic coupling.

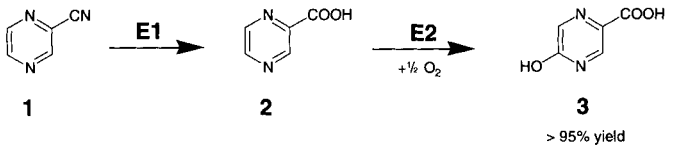
The bacterial strain was found in the Rokko Hot Spring in central Japan. Consequently it is very stable up to temperatures of 60 °C.

The main problem in chemical synthesis coupling of Z-Asp anhydride with



1 = phenylalanine methylester
2 = aspartic acid (protected)
3 = α-aspartame (protected)
E = thermolysin, enzyme from *Bacillus proteolicus*

Figure 19-34. Biocatalytical synthesis of aspartame (HSC, Holland Sweetener Company).



1 = 2-cyanopyrazine
2 = pyrazine-2-carboxylic acid
3 = 5-hydroxypyrazine-2-carboxylic acid
E1/E2 = nitrilase/hydroxylase, whole cells, strain *Agrobacterium* sp. contains both enzymes

Figure 19-35. Biocatalytical synthesis of 5-hydroxypyrazine-2-carboxylic acid (Lonza).

L-Phe-OCH_3 is the by-product formation of β-aspartame. This isomer is of bitter taste and has to be completely removed from the α-isomer. The advantages of the enzymatic route are: (i) No β-isomer is produced, (ii) the enzyme is completely stereoselective, so that racemic mixtures of the substrate or the appropiate enantiomer of the amino acid can be used, (iii) no racemization occurs during synthesis and (iv) the reaction takes place in aqueous media under mild conditions.

19.3.4.11
Hydrolysis of Heterocyclic Nitrile by Nitrilase from *Agrobacterium* sp. (E.C. 3.5.5.1)[94–96]

5-Hydroxypyrazine-2-carboxylic acid (Fig. 19-35) is a versatile building block in the synthesis of new antituberculous agents, e.g. 5-chloro-pyrazine-2-carboxylic acid esters. The regioselective hydroxylation of pyrazine-2-carboxylic acid is catalyzed by a hydroxylase (**E2**, E.C. 1.5.1.13). This second enzyme is also in the applied suspended whole cells from *Agrobacterium* sp. The biomass is separated by ultrafiltration (cutoff 10 kDa) after the biotransformation. 5-Hydroxypyrazine-2-carboxylic acid is precipitated from the permeate by acidification with sulfuric acid to pH 2.5.

In contrast to the biotransformation, the chemical synthesis of 5-substituted pyrazine-2-carboxylic acid leads to a mixture of 5- and 6-substituted pyrazine-carboxylic acids and requires multiple steps.

19.3.5
Formation of C-O Bonds by Lyases

19.3.5.1
Synthesis of Carnitine Catalyzed by Carnitine Dehydratase in Whole Cells (E.C. 4.2.1.89)[8, 46, 97–99]

L-Carnitine is used in infant health, sport and geriatric nutrition. The biotransformation is catalyzed by carnitine dehydratase in whole cells (Fig. 19-36). (*R*)-carnitine is produced with > 99.5% conversion of butyrobetaine and > 99.5% *ee*. The mutant strain has blocked the L-carnitine dehydrogenase and excretes the accumulated product. The purified enzyme could not be used for the biotransformation because of its high instability. Apart from usual batch fermentations, continuous production

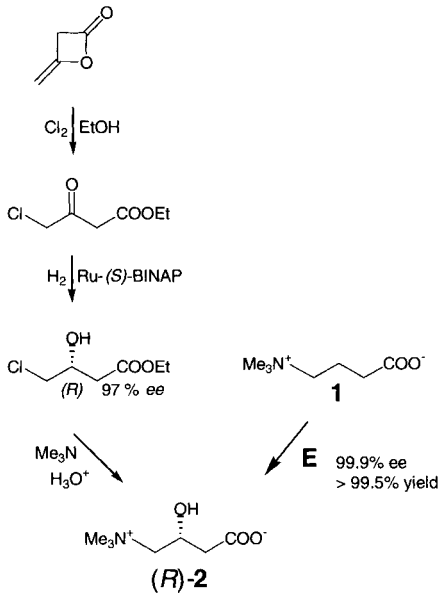


Figure 19-36. Comparison of chemical and biocatalytical synthesis of carnitine (Lonza).

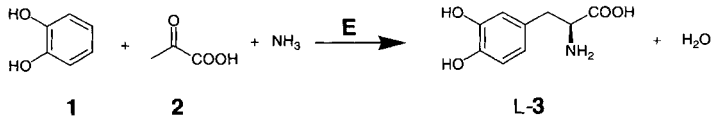
1 = 4-butyro betaine
2 = carnitine
E = carnitine dehydratase, whole cells from *Escherichia coli*

is also feasible since the cells go into a "maintenance state" with high metabolic activity and low growth rate. The cells can be recycled after separation from the fermentation broth by filtration. A chemical resolution process with L-tartaric acid that was developed at Lonza was no longer competitive with the biotechnological route. A more attractive chemical route would be the Ru-BINAP catalyzed asymmetric hydrogenation of 4-chloroacetoacetate (Fig. 19-36). Here an *ee* of 97% is yielded.

19.3.6
Formation of C-N Bonds by Lyases (E.C. 4)

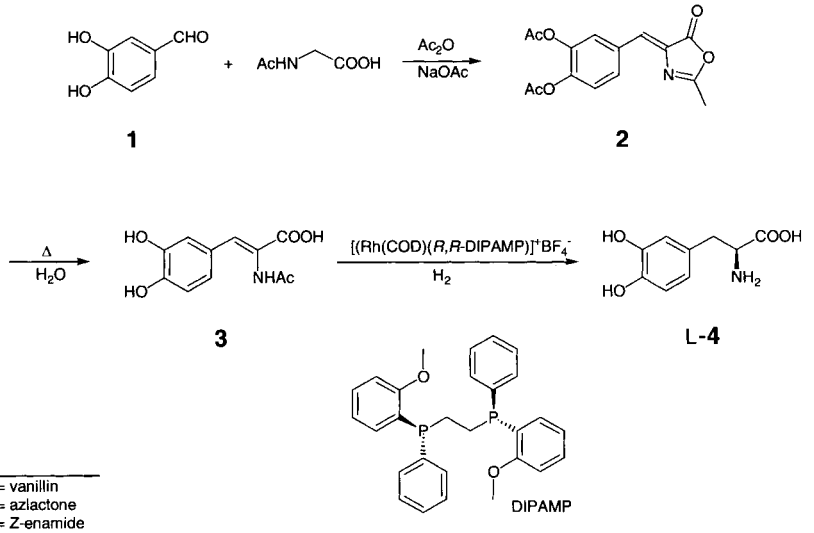
19.3.6.1
Synthesis of L-Dopa Catalyzed by Tyrosine Phenol Lyase from *Erwinia herbicola* (E.C. 4.1.99.2)[100–103]

The product is applied for the treatment of Parkinsonism that is caused by a lack of L-dopamine and its receptors in the brain. L-Dopamine is synthesized in organisms by decarboxylation of L-3,4-dihydroxyphenylalanine (L-dopa). Since L-dopamine cannot pass the blood-brain barrier L-dopa is applied in combination with dopadecarboxylase-inhibitors to avoid formation of L-dopamine outside the brain. Ajinomoto produces L-dopa by this lyase-biotransformation with suspended whole cells in a fed batch reactor on a scale of 250 t a^{-1}. Much earlier, Monsanto has successfully scaled up the chemical synthesis of L-dopa (Fig. 19-38).



1 = catechol
2 = pyruvic acid
3 = dopa
E = tyrosine phenol lyase, whole cells from *Erwinia herbicola*

Figure 19-37. Biocatalytical synthesis of L-dopa (Ajinomoto).



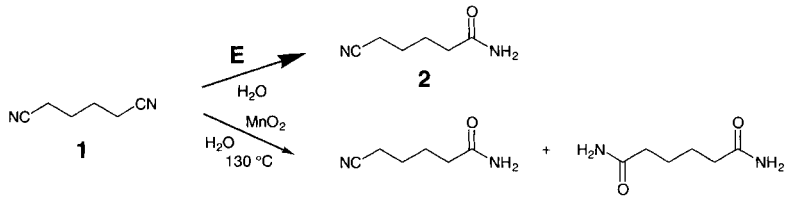
1 = vanillin
2 = azlactone
3 = Z-enamide
3 = dopa

Figure 19-38. Chemical synthesis of L-dopa (Monsanto).

The enantioselective hydrogenation of 3,4-dihydroxy-*N*-acetylamino cinnamic acid is catalyzed by the cationic Rh-biphosphine complex DIPAMP, in which the enantioselectivity is introduced by the chiral phosphine[104, 105]. The hydrogenation proceeds quantitatively with 94 % *ee*. The optically pure L-dopa is separated from the catalyst by crystallization.

19.3.6.2
Synthesis of 5-Cyano Valeramide by Nitrile Hydratase from *Pseudomonas chlororaphis* B23 (E.C. 4.2.1.84)[106, 107]

5-Cyanovaleramide is used as intermediate for the synthesis of the DuPont herbicide azafenidine (Fig. 19-39). The whole cells from *Pseudomonas chlororaphis* are immobilized in calcium alginate beads. The biotransformation itself is catalyzed by a nitrile hydratase that converts a nitrile into the corresponding amide by addition of water. Nitrile hydratases belonging to the enzyme class of lyases (E.C. 4) are not be



1 = adiponitrile
2 = 5-cyano-valeramide
E = nitrile hydratase, whole cells from *Pseudomonas chlororaphis*

Figure 19-39. Comparison of chemical and biocatalytical synthesis of 5-cyano-valeramide (DuPont).

confused with the nitrilases belonging to the class of hydrolases (E.C. 3) that hydrolyze nitriles to the corresponding carbon acids. For strain selection it was important that the cells did not show any amidase activity that would further hydrolyze the amide to the carboxylic acid. The biotransformation is carried out in a two-phase system with pure adiponitrile forming the organic phase. A reaction temperature of 5 °C is chosen, since the solubility of the by-product adipodiamide is only 37–42 mM in 1–1.5 M 5-cyanovaleramide. A batch reactor is preferred over a fixed-bed reactor, because of the lower selectivity to 5-cyanovaleramide that was observed and the possibility of precipitation of adipodiamide and plugging of the column. Excess water is removed at the end of the reaction by distillation. The by-product adipodiamide is precipitated by dissolution of the resulting oil in methanol at > 65 °C. The raw product solution is directly transferred to the herbicide synthesis.

By this method 13.6 tonnes have been produced in fifty-eight repetitive batch cycles with 97 % conversion and 96 % selectivity. This biotransformation was chosen over the chemical transformation because of the higher conversion and selectivity, production of more product per catalyst weight (3 150 kg per kg dry cell weight), and less waste. The catalyst consumption is 0.006 kg per kg product.

19.3.6.3
Synthesis of the Commodity Chemical Acrylamide Catalyzed by Nitrile Hydratase from *Rhodococcus rodochrous* (E.C. 4.2.1.84) [108–112]

Acrylamide (Fig. 19-40) is an important commodity monomer used in coagulators, soil conditioners and stock additives for paper treatment and paper sizing, and for adhesives, paints and petroleum recovering agents.

Since acrylonitrile is the most poisonous of the nitriles, screening for microorganisms was conducted with low molecular weight nitriles instead.

Acrylamide is unstable and polymerizes easily; therefore the process is carried out at a low temperature (5 °C). Although the cells, which are immobilized on polyacrylamide gel, and the contained enzyme are very stable towards acrylonitrile, the starting material has to be fed continuously to the reaction mixture because of inhibition effects at higher concentrations. The biotransformation is started with an

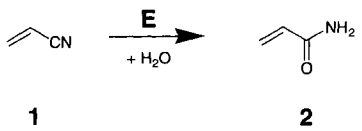


Figure 19-40. Biocatalytical synthesis of acrylamide (Nitto Chemical Industry).

1 = acrylonitrile
2 = acrylamide
E = nitrile hydratase, whole cells from *Rhodococcus erythropolis*

acrylonitrile concentration of 0.11 M and is stopped at an acrylamide concentration of 5.6 M. The process is operated at a capacity of 30 000 t a^{-1}.

This nitrile hydratase acts also on other nitriles with yields of 100%. The most impressive example is the conversion of 3-cyanopyridine to nicotinamide. The product concentration is about 1 465 g L^{-1}. This conversion (1.17 g L^{-1} dry cell mass) can be named "pseudocrystal enzymation", since at the start of the reaction the educt is solid and with ongoing reaction it is solubilized.

The chemical synthesis uses copper salt as catalyst for the hydration of acrylonitrile and has several disadvantages:

- The rate of acrylamide formation is lower than that of acrylic acid formation.
- The double bond of the starting material and the product causes the formation of by-products such as ethylene, cyanohydrin and nitrilotrispropionamide.
- Polymerization occurs.
- Copper needs to be separated from the product (an extra step in the chemical synthesis).

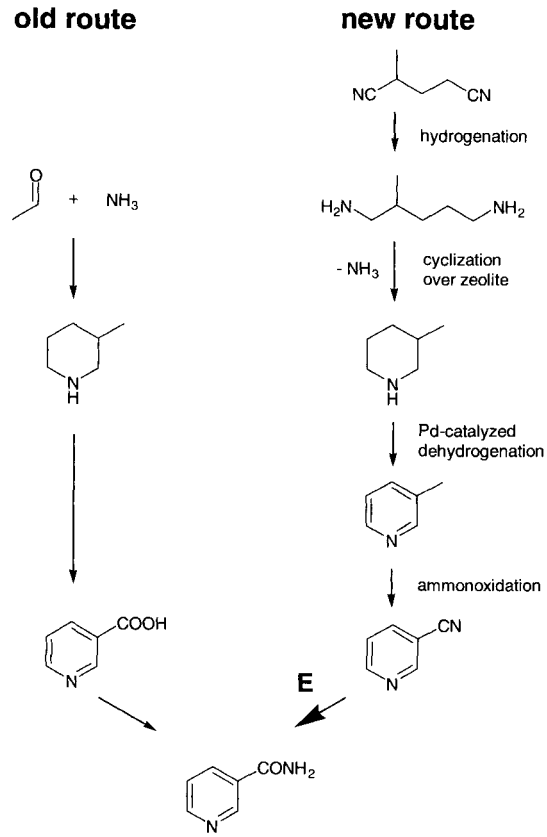
The biotransformation has the advantages that no recovering of unreacted nitrile is necessary since the conversion is 100% and no copper catalyst removal is needed. This is also the first case of a biocatalytic conversion of a bulk fiber monomer.

19.3.6.4

Synthesis of Nicotinamide Catalyzed by Nitrile Hydratase from *Rhodococcus rodochrous* (E.C. 4.2.1.84)[48, 113]

Nicotinamide (vitamin B3) is used as a vitamin supplement for food and animal feed. It is the same strain that is also used in the industrial production of acrylamide (see Sect. 19.3.6.3). The biotransformation is carried out on a scale of 3000 t a^{-1} (Fig. 19-41).

In contrast to the chemical alkaline hydrolysis of 3-cyanopyridine with 4% by-product of nicotinic acid (96% yield) the biotransformation works with absolute selectivity and no acid or base is required. The biotransformation (a continuous process) is operated at low temperature and atmospheric pressure. In contrast to the old synthesis route of nicotinamide at Lonza, the new one is environmentally friendly and safe. There is only one organic solvent used throughout the whole process in four highly selective continuous and catalytic reactions. The process water, NH_3 and H_2 are recycled.



E = nitrile hydratase, whole cells from *Rhodococcus erythropolis*

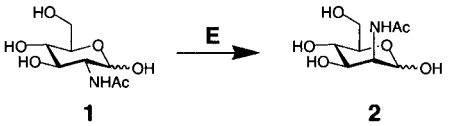
Figure 19-41. Comparison of chemical and biocatalytical synthesis of nicotinamide (Lonza).

19.3.7
Epimerase

19.3.7.1
Epimerization of Glucosamine Catalyzed by Epimerase from *E. coli* (E.C. 5.1.3.8)[114–116]

N-Acetyl-D-mannosamine serves as the *in situ* generated substrate for the synthesis of *N*-acetylneuraminic acid. Since *N*-acetyl-D-mannosamine is quite expensive it is synthesized from *N*-acetyl-D-glucosamine by epimerization at C_2. This biotransformation is integrated into the production of *N*-acetylneuraminic acid (Neu5Ac).

By application of *N*-acylglucosamine 2-epimerase it is possible to start with the inexpensive *N*-acetyl-D-glucosamine instead of *N*-acetyl-D-mannosamine (Fig. 19-42). The epimerase is used for the *in situ* synthesis of *N*-acetyl-D-mannosamine



1 = *N*-acetyl-D-glucosamine
2 = *N*-acetyl-D-mannosamine
E = GlcNAc 2-epimerase, enzyme from *Escherichia coli*

Figure 19-42. Biocatalytical epimerization of glucosamine to mannosamine (Marukin Shoyu).

(ManNAc). Since the equilibrium is on the side of the starting material, the reaction is driven by the subsequent biotransformation of ManNAc together with pyruvate to Neu5Ac.

The *N*-acylglucosamine 2-epimerase is cloned from porcine kidney, transformed and overexpressed in *Escherichia coli*. To reach maximal activitiy, ATP and Mg^{2+} need to be added. Since the whole synthesis is reversible, high GlcNAc concentrations are used.

The chemical epimerization of GlcNAc is used by Glaxo. The equilibrium of the chemical epimerization is on side of *N*-acetyl-D-glucosamine (GlcNAc:ManNAc = 4:1). After neutralization and addition of isopropanol GlcNAc precipitates. In the remaining solution a ratio of GlcNAc:ManNAc = 1:1 is reached. After evaporation to dryness and extraction with methanol the ratio of GlcNAc:ManNAc is shifted to 1:4.

19.4 Some Misconceptions about Industrial Biotransformations

There are a lot of prejudices against biotransformations. The major ones are:

- Biocatalysts are too expensive.
- Biocatalysts only work under mild conditions.

The first prejudice that biocatalysts are too expensive is only partly true. If the cost per mol or per unit weight is calculated they certainly are expensive. For example, penicillin amidase costs \$ 10 000/kg on a bulk scale. On the other hand the cost contribution of penicillin amidase in the "splitting" of penicillin G is only \$ 1/kg of product[117]. In the case of L-aspartic acid production the cost contribution of aspartase is even lower, \$ 0.1/kg. This demonstrates that it is not the absolute catalyst cost but the cost contribution of the catalyst to the final product cost that has to be considered and compared. This is also true for chemical catalysts; e.g., the bulk price of BINAP is \$ 40 000/kg[117]. Important parameters influencing the cost contribution are the total turnover number (mol product/mol catalyst) and the turnover frequency (mol product/mol catalyst and unit time).

The second prejudice, that biocatalysts only work in an aqueous phase with low concentrations of starting material is also only partly true. The natural environment

Table 19-1. Highest concentrations applied in industrial biotransformations.

EC	enzyme	substrate	concentration	medium
1.1.99.21	D-Sorbitol dehydrogenase	1-Amino-D-sorbitol (N-protected)	**1.00 M**	Aqueous
4.2.1.2	Fumarase	Fumaric acid	**1.00 M**	Aqueous
3.4.21.62	Subtilisin	Phenylalanine isopropylester	**1.20 M**	Aqueous
3.1.1.3	Lipase	1-Phenylethylamine	**1.65 M**	MTBE
3.5.2.6	β-Lactamase	γ-Lactam	**1.83 M**	Aqueous
4.2.1.84	Nitrile hydratase	Adiponitrile	**2.01 M**	Aqueous/ organic
4.3.1.1	L-Aspartase	Fumaric acid	**2.00 M**	Aqueous
4.1.1.12	Aspartate β-decarboxylase	Aspartic acid	**2.50 M**	Aqueous
3.1.1.25	Lactonase	Pantolactone	**2.69 M**	Aqueous
3.1.1.3	Lipase	Palmitic acid	**3.10 M**	2-Propanol
3.1.1.3	Lipase	Cyclopentenylester	**4.16 M**	Aqueous/ organic
4.2.1.84	Nitrile hydratase	Acrylonitrile	**5.60 M (product)**	Aqueous
4.3.1.5	L-Phenylalanine ammonia-lyase	*trans*-Cinnamic acid	**9.31 M (NH_3)**	Aqueous

is in general the aqueous phase and ambient temperature. But the examples described above demonstrate that biocatalysts can be also applied in emulsions or even pure organic solvents (Table 19-1). Here, moreover, very high concentrations are reached, e.g. in the case of acrylamide up to 5.6 M.

19.5 Outlook

Despite the progress biocatalysis has made in the last few years its potential is still increasing. By improved screening methods new catalysts will be detected and made available in large amounts by cloning and overexpression. Directed evolution will be used to improve properties such as stability or selectivity[118, 119]. Metabolic engineering will be used to analyze and remove bottlenecks in the metabolism or to create novel biocatalysts[120].

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Karlheinz Drauz and Herbert Waldmann
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20
Tabular Survey of Commercially Available Enzymes

Peter Rasor

Enzymes are catalysts. Nature has designed them to perform specific tasks necessary for the survival of the organism producing the enzyme. The organic chemist tends to name enzymes "biocatalysts" which means nothing more than catalysts of *bio*logical origin.

These biocatalysts bring some confusion to the well-structured world of organic chemistry:

- the names are unfamiliar,
- each enzyme has a variety of names which are all used making it as difficult as distinguishing characters in a Russian novel (e.g. Penicillin G-amidase and Penicillin acylase),
- when it comes to microogranisms or plants, the origin of enzymes is described in Latin (type face italic),
- in order to add to the confusion, the names of microorganisms may change over time, for example *Pseudomonas cepacia* is now *Burkholderia cepacia*, *Candida cylindracea* is *Candida rugosa*,
- even mammalian sources can be described differently – esterase from *hog* liver or *pig* liver, but lipase from *porcine* pancreas (type face not italic).

For identifying synonyms or finding out the correct name of an enzyme, the Enzyme Nomenclature Database (EC database) can be searched or downloaded under http://www.expasy.ch/enzyme/.

If the chemist is still not confused and has mastered this hurdle, the manufacturers or suppliers introduce brand names for marketing reasons, and may even change names once in a while. Additionally, not every supplier gives full information on the origin of the biocatalyst and may use old names of microorganisms while other suppliers already use new names.

Furthermore, the same biocatalyst by description may behave differently in a specific reaction: for example, lipase from *Candida rugosa* from Amano (Lipase AY) differs from Lipase MY or OF from Meito Sangyo with respect to activity and stereoselectivity because it consists of a number of catalytically active species which differ depending on the production strain used and thus, on the manufacturer.

Table 20-1. Abbreviation of most commonly used biocatalysts

Abbreviation	Lipase from	Abbreviation	Lipase from
ANL	*Aspergillus niger*	PcamL	*Penicillium camembertii*
BCL (PCL)	*Burkholderia cepacia* (formerly *Pseudomonas cepacia*)	PFL	*Pseudomonas fluorescens*
CAL	*Candida antarctica*	PfragiL	*Pseudomonas fragi*
CAL-A	*Candida antarctica*, type A	PPL	*Porcine pancreas*
CAL-B	*Candida antarctica*, type B	ProqL	*Penicillium roquefortii*
CLL	*Candida lipolytics*	PSL	*Pseudomonas* sp.
CRL (CCL)	*Candida rugosa* (formerly *C. cylindracea*)	RML (MML)	*Rhizomucor miehei* (formerly *Mucor miehei*)
CVL	*Chromobacterium viscosum* (identical to *Pseudomonas glumae*)	ROL	Rhizopus oryzae (other names: RNL – *Rhizopus niveus*, RDL – *Rhizopus delemar*, RJL – *Rhizopus javanicus*)
GCL	*Geotrichum candidum*	TLL (HLL)	*Thermomyces lanuginosa* (formerly *Humicola lanuginosa*)
Abbreviation	**Esterase from**	**Abbreviation**	**Alcohol dehydrogenase from**
PLE	Pig liver	YADH	Yeast
		TBADH	*Thermoanaerobium brockii*
		HLADH	Horse liver

Since the full enzyme name according to the EC nomenclature is rather long, the most commonly used enzymes have gotten abbreviations. For esterases and lipases there are certain rules: in most cases, the first (two or three) letters characterize the source, the last the type of enzyme (E for esterase, L for lipases) (see Table 20-1). Alcohol dehydrogenases are treated similarly.

All this may explain why many publications give only incomplete information on the exact type of enzyme used in the work described and why many references to enzymes are simply wrong. The author strongly recommends to provide at least the following information:

Parameter	Example 1	Example 2
Name of the product	Lipase Type XIII	CHIRAZYME L-2, lyo
Description (if the name of the product is a brand name or non-descriptive)	Lipase from *Pseudomonas* sp.	Lipase from *Candida antarctica*, type B
Formulation	Powder	Powder
Manufacturer	Sigma	Roche Diagnostics

Table 20-2. Available screening sets

Enzyme type	Company
Alcohol dehydrogenases	ThermoGen, BioCatalytics
Esterases & lipases	Altus, Fluka, Roche, ThermoGen
Nitrilases	BioCatalytics
Proteases	Altus
Transaminases (aminotransferases)	BioCatalytics

In the laboratory protocol, lot. no. and activity (incl. assay no. or assay conditions) must be recorded as well in order to track variation in results because of lot to lot inconsistency.

Every development of a biocatalytic reaction starts with a screening for the most appropriate enzyme. Some companies offer screening sets (or kits) containing the most commonly used enzymes (Table 20-2). Some Sets are single use (Altus, ThermoGen) while others contain enough material to perform depending on the scale 5–20 experiments (BioCatalytics, Fluka, Roche). These sets may include enzymes available on industrial scale or on research scale only.

The following companies offer screening set/kits for quick enzyme selection (Table 20-2). While some companies include only industrial scale enzymes, others contain enzymes only available at lab quantities. Diversa Co. offers an enzyme subscription program for lipases, esterases, nitrilases, cellulases, glycosidases, phosphatases, and transaminases (aminotransferases).

Some enzymes of Novozymes A/S (formerly Novo Nordisk A/S) were widely distributed on an experimental stage (SP nnn). Table 20-3 lists the most important

Table 20-3. List of experimental enzymes by Novozymes, current products names and suppliers

Old name	Characterization	Current brand name	Availability
SP 361 SP 409	Immobilized enzyme mixture from Rhodococcus sp. containing nitrilase, nitril hydratase,esterase, epoxide hydrolase and amidase activity		discontinued
SP 382	Immobilized lipase from Candida antarctica, containing type A & B		discontinued
SP 435	Immobilized lipase from Candida antarctica, type B, rec. in Aspergillus oryzae	Novozym 435 CHIRAZYME L-2, Carrier 2	Novo-Nordisk Roche Diagnostics
SP 523	Lipase powder from Thermomyces lanuginosus (formerly Humicola lanuginosa)	CHIRAZYME L-8, lyo	Roche Diagnostics
SP 524	Lipase powder from Rhizomucor miehei, rec. in Aspergillus oryzae	CHIRAZYME L-9, lyo	Roche Diagnostics
SP 525	Lipase powder from Candida antarctica, type B, rec. in Aspergillus oryzae	CHIRAZYME L-2, lyo	Roche Diagnostics
SP 526	Lipase from Candida antarctica, type A, rec. in Aspergillus oryzae	CHIRAZYME L-5, lyo	Roche Diagnostics

Table 20-4. Enzyme producers/suppliers and brief characterization

Company	Adress	Tel./Fax/Email/WWW	Focus/Characterization[1]
Altus	Altus Biologics Inc. 625 Putnam Avenue Cambridge, MA 02139–4807 USA	Tel.: +1 (617) 299-2900 Fax: +1 (617) 299–2999 Email: info@altus.com http://www.altus.com	Manufacturer of stabilized enzymes for use in industrial, biocatalytical, diagnostic, and medicinal applications. No enzyme production itself. Biocatalytical process development.
Amano	Amano Pharmaceutical Co., Ltd. 2–7, 1-chome, Nishiki Naka-ku, Nagoya, 460–8630 Japan	Tel.: +81 (52) 211-3032 Fax: +81 (52) 211–3054 http://www.amano-enzyme.co.jp	Specialty enzyme producer for industrial, biocatalytical, diagnostic, and medicinal applications.
Asahi Chemical Industry Co.	Diagnostics Division Hibiya-Mitsui Building 1–2 Yurakucho 1-chome, Chiyoda-ku Tokyo 100–8440 Japan	Tel.: +81 (3) 3259-5776 Fax: +81 (3) 3259–5741 Email: shindan@ml.asahi-kasei.co.jp http://www.asahi-kasei.co.jp	Speciality enzyme producer for diagnostic and medicinal applications.
Biocatalysts	Biocatalysts Ltd Main Avenue, Treforest Industrial Estate Pontypridd, Wales, CF37 5UD United Kingdom	Tel.: +44 (0) 1443843712 Fax: +44 (0) 1443841214 Email: sales@biocats.com http://www.biocatalysts.com	Producer and distributor of enzymes for use in industrial and diagnostic applications.
BioCatalytics	BioCatalytics Inc. 39 Congress Street, Suite 303 Pasadena, CA 91105–3022 USA	Tel.: +1 (626) 229–0588 Fax: +1 (626) 535–9465 Email: info@biocatalytics.com http://www.biocatalytics.com	Biocatalytical process development. Experimental enzymes for biocatalysis. Limited production capacity. Distributor for Roche in USA and Canada.
Biozyme Laboratories International Ltd.	USA and Canada: 9939 Hibert Street Suite 101 San Diego, CA 92131–1029 USA	Tel.: +1 (858) 549-4484 or (800) 423-8199 Fax: (858) 549–0138 Email: bioinfo@biozyme.com	Speciality enzyme producer for diagnostic and medicinal applications
	All other countries: Biozyme Laboratories Ltd. Unit 6, Gilchrist Thomas Estate Blaenavon, South Wales, NP4 9RL United Kingdom	Tel.: (+44) 1495790678 Fax: (+44) 1495791780 Email: info@biozyme.co.uk http://www.biozyme.com/	
Calbiochem. Co., CN Biosciences	Calbiochem-Novabiochem Corporation 10394 Pacific Center Court San Diego, CA 92121 Mailing Address: P.O. Box 12087 La Jolla, CA 92039–2087 USA	Tel.: +1 (858) 4509600 or (800) 8543417 Fax: +1 (858) 4533552 Email: orders@calbiochem.com. technical@calbiochem.com http://www.calbiochem.com http://www.cnbi.com	Supplier of enzymes and biochemicals on research scale. Focus on life science, not biocatalysis.

Table 20-4. (cont.).

Company	Adress	Tel./Fax/Email/WWW	Focus/Characterization[1]
Diversa	Diversa Corporation 4955 Directors Place San Diego, CA 92121–1609 USA	Tel.: +1 (858) 526–5000 Fax: +1 (858) 526–5551 Email: information@diversa.com http://www.diversa.com	Discovery and development of industrial enzymes. No general biocatalyst portfolio.
DSM Gist-Brocades	DSM Food Specialties P.O. Box 1 2600 MA Delft The Netherlands	Tel.: +31 (15) 2793474 Fax: +31 (15) 2793540 http://www.dsm.nl/dfs/	Enzyme producer for industrial applications (feed & food).
Fluka	see Sigma-Aldrich Fluka		
Genencor	Genencor International, Inc. 200 Meridian Centre Blvd. Rochester, NY 14618–3916 USA	Tel.: +1 (716) 256-5200 Fax: +1 (716) 256–6952 Email: ysmith@genencor.com http://www.genencor.com	Enzyme producer for industrial applications.
Jülich Enzyme Products	Juelich Enzyme Products GmbH Karl-Heinz-Beckurts-Str. 13 D-52428 Jülich Germany	Tel.: +49 (2461) 348188 Fax: +49 (2461) 348186 E-mail: juelichep@aol.com http://www.juelich-enzyme.com	Experimental enzymes for biocatalysis. Limited enzyme production capacity.
Lee Scientific	Lee Scientific Inc. 2924 Mary Ave. St. Louis, MO 63144 USA	Tel.: +1 (314) 968-1091 Fax: +1 (314) 968–9851 Email: burtonlee@leescientific.com http://www.leescientific.com/	Specialty enzyme producer. Focus on life science and diagnostics. Some biocatalysts.
Meito Sangyo Co. Ltd.	Fine Chemicals Dept. Meito Sangyo Co. Ltd. Sankeido Bldg., 4–3-15, Muromachi, Nihonbashi Chuo-ku, Tokyo 103–0022 Japan	Tel.: +81 (3) 3242-1795 Fax: +81 (3) 3242–1792 Email: jdt02625@nifty.ne.jp	Producer and distributor of enzymes for use in industrial and diagnostic applications.
Novozyme A/S	Europe, Middle East & Africa: Novozymes France S.A. Immeuble Challenge 92 79, Avenue Frantois Arago 92017 Nanterre Cedex, France	http://www.novozymes.com Tel.: +33 146 140746 Fax: +33 146 140766	Largest enzyme producer for industrial applications. Distribution agreement with Roche for chiral organic synthesis market.
	Latin America: Novozymes Latin America Limited Rua professor Francisco Ribeiro 683 CEP 83707–660 – Araucaria – Parana Brazil	Tel.: +55 416411000 Fax: +55 416431443	

Table 20-4. (cont.).

Company	Adress	Tel./Fax/Email/WWW	Focus/Characterization[1]
	USA: Novozymes North America Inc. 77 Perry Chapel Church Road Franklinton, N.C. 27525 Postal Address: State Road 1003 P.O. BOX 576 Franklinton, NC 27525	Tel.: +1 919 494 3000 Fax: +1 919 494 3450	
	Asia Pacific, Hong Kong: Novozymes Asia Pacific Regional Office 7/F Chinachem Century Tower 178 Gloucester Road, Wanchai	Tel.: +852 2519 3380 Fax: +852 2877 0659	
Recordati S.p.A.	Via Matteo Civitali, 1 20148 Milan Italy	Tel.: +39 (02) 487871 http://www.recordati.it	Manufacturer of industrial enzymes for beta-lactam antibiotics.
Roche Diagnostics	Roche Diagnostics GmbH Roche Molecular Biochemicals Sandhofer Str. 116 68298 Mannheim Germany	Tel.: +49 (621) 7598593 Fax: +49 (621) 7598986 Email: ute.hill@roche.com http://indbio.roche.com	Speciality enzyme producer for industrial, biocatalytical, diagnostic, and medicinal applications. Broad range of enzymes.
	USA & Canada: Refer to BioCatalytics Inc.		
Seravac	Seravac USA, Inc. 13220 Evening Creek Drive San Diego, CA 92128 USA	Tel.: +1 (858) 679–4050 or (800) 679–4050 Fax: (858) 679–1438 Email: enzymes@seravac.com http://www.seravac.com	Speciality enzyme producer for diagnostic and medicinal applications.
Sigma-Aldrich Fluka (SAF)	Sigma Co. 3050 Spruce Street St. Louis, MO 63103 Mail: P. O. Box 14508 St. Louis, MO 63178 USA	Tel.: (314) 771–5765 Fax: (314) 771–5757 Email: sigma@sial.com http://www.sigma-aldrich.com	Manufacturer and distributor of enzymes and biochemicals on research scale. Very broad range of enzymes (Sigma and Fluka). Limited range of biocatalysts at Aldrich. Within the group, Fluka has the focus on biocatalysts on research scale. Production of selected enzymes up to medium scale.
	Fluka Chemical LLC. Industriestrasse 25 CH-9471 Buchs Mail: P. O. Box 260 CH-9471 Buchs Switzerland	Tel.: +41 (81) 755 28 28 Fax: +41 (81) 756 54 49 EMail: fluka@sial.com http://www.sigma-aldrich.com	
ThermoGen	ThermoGen, Inc. 2501 Davey Road Woolridge, IL 60517 USA	Tel.: +1 (630) 783-4600 Fax: +1 (630) 783–4909 info@thermogen.com http://www.thermogen.com	Enzyme discovery. Limited enzyme production capacity. Biocatalytical process development.

Table 20-4. (cont.).

Company	Adress	Tel./Fax/Email/WWW	Focus/Characterization[1]
Toyobo Co. Ltd.	Toyobo Co. Ltd. Biochemical Operations Department 17–9 Nihonbashi Koami-cho Chuo-ku Tokyo 103–8530 Japan	Tel.: +81 (3) 3660–4819 Fax: +81 (3) 3660–4951 EMail: toshiro–kikuchi@bio.toyobo.co.jp http://www.toyobo.co.jp/e/	Speciality enzyme producer for diagnostic and medicinal applications.
Unitica Ltd.	Medical Products Division Unitika Ltd. 4–1-3, Kyutaro-machi, Chuo-ku, Osaka 541–8566 Japan	Tel.: +81(6) 6281–5021 Fax: +81 (6) 6281–5256 Email : medical@unitika.co.jp http://www.unitika.co.jp/ home–e.htm	Specialty enzyme producer for diagnostic and medicinal applications.
Wako Pure Chemicals Industries, Ltd.	1–2, Doshomachi 3-Chome, Chuo-Ku, Osaka 540–8605 Japan	Tel.: +81 (6) 6203-3741 Fax: +81 (6) 6222–1203 http://search.wako-chem.co.jp	Manufacturer and distributor of enzymes and biochemicals on research scale. Focus on life science, not biocatalysis.
Worthington Biochemical	Worthington Biochemical Corp. 730 Vassar Ave Lakewood, NJ 08701	Tel.: +1 (732) 942–1660 Fax: +1 (732) 942–9270 http://www.worthington-biochem.com/	Manufacturer and distributor of enzymes and biochemicals. Focus on life science and diagnostics.

1 Industrial applications include detergents, feed and food, pulp & paper, etc.

enzymes and gives the current brand names, wherever possible. Some enzymes have been discontinued at Novozymes but replacements are available from Roche Diagnostics (CHIRAZYME product line).

Catalytic antibodies are not yet widely available. Aldrich is offering two aldolase monoclonal antibodies.

The major enzyme producers and/or suppliers are listed and briefly characterized in Table 20-4. The author is aware that the list of enzyme producers is not complete.

The author has made the attempt to list enzymes that are commercially available (Table 20-5) and thus can be used in biocatalysis. He knows that the list is incomplete and therefore, the reader should not rely solely on this list but rather check the suppliers listed in Table 20-4. Enzyme manufacturers also update their product portfolio continuously, so this list probably needs updating before the book is even in print.

A special word is necessary with respect to the Sigma-Aldrich-Fluka conglomerate: Fluka has taken the lead in biocatalysis, while Sigma serves mostly the life science market. Especially since the Sigma catalog is a book in itself, only enzymes from Fluka are listed. The reader should be aware that the majority of enzymes is available from Sigma as well, and with respect to enzymes not typically used in biocatalysis, the portfolio may be even greater.

Explanations to Table 20-5:
The table is sorted by the ***EC number.*** In most cases the number is given in the

respective chapter and can be used to find the enzyme in the table. If the EC no. is not known, at least the general reaction of the enzyme class is given according to the EC nomenclature.

Underneath the ***EC name***, ***synonyms*** are given. The ***general reaction*** according EC nomenclature is denoted too. Afterwards, the ***product (enzyme) names*** are listed, one entry for each ***manufacturer*** per enzyme. If the product is sold under a ***brand name***, this name is listed too. In one enzyme class, the entries are sorted by ***origin***.

The ***availability*** is characterized in three categories: lab, pilot and industrial scale. It refers to the scale with respect to biocatalytical reactions. The author recognized that this categorization is somewhat arbitrary and in some cases may not be correct because the actual production scale is not generally known. Hopefully, though, it will prove to be useful as a rough guide.

Enzyme producers are devoted to certain markets like food & feed, detergents, diagnostics or research. Large enzyme producers such as Novozymes, Genencor or DSM Gist-brocades are categorized as "industrial", specialty enzyme producers like Amano, Asahi or Roche Diagnostics serve various markets and thus, scale varies from pilot to industrial. Since the enzyme demand for diagnostics is much lower than for biocatalysis, typical diagnostic enzymes are labeled as "pilot" although the manufacturing process is certainly standardized and therefore, could be call "industrial" as well. Companies serving the life sciences market (e.g. Sigma-Aldrich Fluka, Roche Diagnostics) have manufacturing capacities from small ("lab") scale to medium scale (here termed as "pilot"). It should also be recognized that the term "pilot scale" in a context other than this table has a different meaning when comparing for example Sigma, Roche Diagnostics, and Novozymes.

Table 20.5. Commercially available enzymes.

Oxidoreductases.	**1.1.1.-**
Acting on the CH-OH group of donors.	
With NAD(+) or NADP(+) as acceptor.	
Alcohol Dehydrogenase Screening Kit; Origin: microorganism, rec. in E. coli ThemoGen: ThermoCat Alcohol Dehydrogenase Kits	Lab
Ketoreductase, broad-range; Origin: microorganism, rec. in E. coli BioCatalytics: KRED-1001	Lab
Ketoreductase, broad-range; Origin: microorganism, rec. in E. coli BioCatalytics: KRED-1002	Lab
Ketoreductase, broad-range; Origin: microorganism, rec. in E. coli BioCatalytics: KRED-1003	Lab
Ketoreductase, broad-range; Origin: microorganism, rec. in E. coli BioCatalytics: KRED-1004	Lab
Ketoreductase, broad-range; Origin: microorganism, rec. in E. coli BioCatalytics: KRED-1005	Lab
Ketoreductase, broad-range; Origin: microorganism, rec. in E. coli BioCatalytics: KRED-1006	Lab
Ketoreductase, broad-range; Origin: microorganism, rec. in E. coli BioCatalytics: KRED-1007	Lab
Ketoreductase, broad-range; Origin: microorganism, rec. in E. coli BioCatalytics: KRED-1008	Lab
Cholesterol Dehydrogenase; Origin: Nocardia sp. Amano: Amano 5 [CHDH-5]	Pilot
7-Hydroxysteroid Dehydrogenase; Origin: Pseudomonas sp. Asahi	Pilot
Alcohol dehydrogenase.	**1.1.1.1**
Aldehyde reductase.	An alcohol + NAD(+) = an aldehyde or ketone + NADH.
Alcohol Dehydrogenase; Origin: Candida parapsilosis Jülich Enzyme Products	Lab
Alcohol Dehydrogenase; Origin: horse liver Fluka	Lab
Alcohol Dehydrogenase; Origin: microorganisms Biocatalysts: Sec ADH 300	Lab
Alcohol Dehydrogenase; Origin: Rhodococcus erythropolis Jülich Enzyme Products	Lab
Alcohol Dehydrogenase; Origin: yeast Biozyme	Pilot
Alcohol Dehydrogenase; Origin: yeast Fluka	Pilot
Alcohol Dehydrogenase; Origin: yeast Roche Diagnostics: Alcohol Dehydrogenase (YADH), lyo.	Pilot
Alcohol Dehydrogenase Origin: yeast Roche Diagnostics: Alcohol Dehydrogenase (YADH), susp.	Pilot
Alcohol Dehydrogenase; Origin: Zymomonas mobilis Unitika	Pilot
Alcohol dehydrogenase (NADP+).	**1.1.1.2**
Aldehyde reductase (NADPH).	An alcohol + NADP(+) = an aldehyde + NADPH.
Alcohol Dehydrogenase; Origin: Lactobacillus kefir Fluka	Pilot

Table 20.5. (cont.).

Alcohol Dehydrogenase; Origin: Lactobacillus kefir
Jülich Enzyme Products — Lab

Alcohol Dehydrogenase; Origin: Thermoanaerobium brockii
Fluka — Pilot

Acetoin dehydrogenase. — **1.1.1.5**
Diacetyl reductase. — Acetoin + NAD(+) = diacetyl + NADH.

Acetoin Dehydrogenase; Origin: Lactobacillus kefir
Fluka — Lab

Diketone Reductase; Origin: Lactobacillus kefir
Jülich Enzyme Products — Lab

Glycerol dehydrogenase. — **1.1.1.6**
Glycerol + NAD(+) = glycerone + NADH.

Glycerol Dehydrogenase; Origin: Bacillus megaterium
Asahi — Pilot

Glycerol Dehydrogenase; Origin: Geotrichum candidum
Fluka — Lab

Glycerol Dehydrogenase; Origin: Klebsiella pneumoniae (formerly Enterobacter aerogenes)
Roche Diagnostics: Glycerol Dehydrogenase — Lab

Glycerol Dehydrogenase (GlDH); Origin: microorganisms
Unitika — Pilot

Glycerol-3-phosphate dehydrogenase (NAD+). — **1.1.1.8**
Sn-glycerol 3-phosphate + NAD(+) = glycerone phosphate + NADH.

Glycerol-3-phosphate Dehydrogenase; Origin: rabbit muscle
Fluka — Lab

L-iditol 2-dehydrogenase. — **1.1.1.14**
Polyol dehydrogenase. Sorbitol dehydrogenase. — L-iditol + NAD(+) = L-sorbose + NADH.

Sorbitol Dehydrogenase (SorDH); Origin: microorganisms
Unitika — Pilot

Sorbitol Dehydrogenase; Origin: sheep liver
Fluka — Lab

Sorbitol Dehydrogenase; Origin: sheep liver
Roche Diagnostics: Sorbitol Dehydrogenase (SDH) — Lab

L-lactate dehydrogenase. — **1.1.1.27**
L-lactic acid dehydrogenase. L-lactic dehydrogenase. — (S)-lactate + NAD(+) = pyruvate + NADH.

L-Lactate dehydrogenase; Origin: beef heart
Biozyme — Pilot

L-Lactate Dehydrogenase; Origin: bovine heart
Fluka — Lab

L(+)-Lactate Dehydrogenase; Origin: hog muscle
Roche Diagnostics: L(+)-Lactate Dehydrogenase (L-LDH) — Pilot

L-Lactate dehydrogenase; Origin: pig heart
Biozyme — Pilot

L(+)-Lactate Dehydrogenase; Origin: pig muscle
Roche Diagnostics: L(+)-Lactate Dehydrogenase (L-LDH) — Pilot

L-Lactate dehydrogenase; Origin: pig muscle
Biozyme — Pilot

Table 20.5. (cont.).

L-Lactate dehydrogenase; Origin: rabbit muscle
Biozyme — Pilot

L-Lactate Dehydrogenase; Origin: rabbit muscle
Fluka — Lab

Lactate Dehydrogenase; Origin: Staphylococcus sp.
Amano: Amano 3 [LDH-3] — Pilot

D-lactate dehydrogenase. **1.1.1.28**
D-lactic acid dehydrogenase. D-lactic dehydrogenase. (R)-lactate + NAD(+) = pyruvate + NADH.

D-Lactate Dehydrogenase; Origin: Lactobacillus leichmanii
Fluka — Lab

D(-)-Lactate Dehydrogenase; Origin: Lactobacillus leichmannii
Roche Diagnostics: D(-)-Lactate Dehydrogenase (D-LDH) — Pilot

D-Lactate Dehydrogenase; Origin: microorganisms
Toyobo — Pilot

D-Lactate Dehydrogenase; Origin: microorganisms
Unitika: D-Lactate Dehydrogenase (D-LDH) — Pilot

3-hydroxybutyrate dehydrogenase. **1.1.1.30**
D-beta-hydroxybutyrate dehydrogenase. (R)-3-hydroxybutanoate + NAD(+) = acetoacetate + NADH.

3-Hydroxybutyrate Dehydrogenase
Asahi — Pilot

D-3-Hydroxybutyrate Dehydrogenase; Origin: Pseudomonas sp.
Toyobo — Pilot

3-Hydroxybutyrate Dehydrogenase; Origin: Rhodobacter sphaeroides (formerly Rhodopseudomonas sphaeroides)
Roche Diagnostics: 3-Hydroxybutyrate Dehydrogenase (3-HBDH), Grade II — Lab

3-Hydroxybutyrate Dehydrogenase; Origin: Rhodopseudomonas spheroides
Fluka — Lab

Malate dehydrogenase. **1.1.1.37**
Malic dehydrogenase. (S)-malate + NAD(+) = oxaloacetate + NADH.

Malate Dehydrogenase; Origin: microorganisms
Toyobo — Pilot

Malate Dehydrogenase; Origin: microorganisms
Unitika: Malate Dehydrogenase (MDH) — Pilot

Malate dehydrogenase; Origin: pig heart
Biozyme — Pilot

Malate Dehydrogenase; Origin: porcine heart
Fluka — Lab

Malate Dehydrogenase; Origin: Thermus sp.
Amano: Amano 3 [MDH-3] — Pilot

Isocitrate dehydrogenase (NADP+). **1.1.1.42**
Oxalosuccinate decarboxylase. IDH. Isocitrate + NADP(+) = 2-oxoglutarate + CO(2) + NADPH.

Isocitrate Dehydrogenase; Origin: porcine heart
Fluka — Lab

Isocitrate Dehydrogenase; Origin: porcine heart
Fluka — Lab

Table 20.5. (cont.).

Phosphogluconate dehydrogenase (decarboxylating). **1.1.1.44**

Phosphogluconic acid dehydrogenase. 6-phosphogluconic dehydrogenase. 6-phosphogluconic carboxylase. 6PGD.

6-phospho-D-gluconate + NADP(+) = D-ribulose 5-phosphate + CO(2) +NADPH.

6-Phosphogluconate Dehydrogenase (6PGDH); Origin: Thermoactinomces intermedius
Unitika — Pilot

6-Phosphogluconic Dehydrogenase; Origin: Torula yeast
Fluka — Lab

6-Phosphogluconic Dehydrogenase; Origin: yeast
Fluka — Lab

Glucose 1-dehydrogenase. **1.1.1.47**

Beta-D-glucose + NAD(P)(+) = D-glucono-1,5-lactone + NAD(P)H.

Glucose Dehydrogenase; Origin: Bacillus megaterium
Fluka — Pilot

Glucose Dehydrogenase; Origin: Bacillus sp.
Amano: Amano 2 [GLUCDH-2] — Pilot

Glucose Dehydrogenase; Origin: Cryptococcus uniguttulatus
Asahi — Pilot

Glucose Dehydrogenase; Origin: microorganisms
Toyobo — Pilot

Glucose-6-phosphate 1-dehydrogenase. **1.1.1.49**

G6PD.

D-glucose 6-phosphate + NADP(+) = D-glucono-1,5-lactone 6-phosphate +NADPH.

Glucose-6-Phosphate Dehydrogenase
Asahi — Pilot

Glucose-6-Phosphate Dehydrogenase (G6PDH); Origin: Bacillus stearothermophilus
Unitika — Pilot

Glucose-6-phosphate Dehydrogenase; Origin: baker's yeast
Fluka — Pilot

Glucose-6-phosphate dehydrogenase; Origin: Leuconostoc mesenteroides
Biozyme — Pilot

Glucose-6-phosphate Dehydrogenase; Origin: Leuconostoc mesenteroides
Fluka — Pilot

Glucose-6-Phosphate Dehydrogenase; Origin: Leuconostoc mesenteroides
Toyobo — Pilot

Glucose-6-phosphate Dehydrogenase; Origin: Leuconostoc mesenteroides
Roche Diagnostics: Glucose-6-phosphate Dehydrogenase (G6P-DH), susp. — Pilot

Glucose-6-phosphate Dehydrogenase; Origin: Leuconostoc mesenteroides, rec. in E. coli
Roche Diagnostics: Glucose-6-phosphate Dehydrogenase (G6P-DH), lyo. — Pilot

Glucose-6-phosphate Dehydrogenase; Origin: Torula yeast
Fluka — Lab

Glucose-6-phosphate dehydrogenase; Origin: yeast
Biozyme — Pilot

Glucose-6-phosphate Dehydrogenase; Origin: yeast
Fluka — Pilot

Glucose-6-phosphate Dehydrogenase; Origin: yeast
Fluka — Pilot

Glucose-6-phosphate Dehydrogenase; Origin: yeast
Roche Diagnostics: Glucose-6-phosphate Dehydrogenase (G6P-DH), lyo. — Pilot

Table 20.5. (cont.).

Glucose-6-Phosphate Dehydrogenase (G6PDH); Origin: Zymomonas mobilis
Unitika — Pilot

3-alpha-hydroxysteroid dehydrogenase (B-specific). — **1.1.1.50**
Hydroxyprostaglandin dehydrogenase. 3-alpha-HSD. — Androsterone + NAD(P)(+) = 5-alpha-androstane-3,17-dione + NAD(P)H.

3-Hydroxysteroid Dehydrogenase
Asahi — Pilot

3-alpha-Hydroxysteroid Dehydrogenase (3alphaHSDH); Origin: microorganisms
Unitika — Pilot

3-alpha-Hydroxysteroid Dehydrogenase; Origin: Pseudomonas testosteroni
Fluka — Lab

Xanthine dehydrogenase. — **1.1.1.204**
Xanthine oxidoreductase. — Xanthine + NAD(+) + H(2)O = urate + NADH.

Xanthine Dehydrogenase
Asahi — Pilot

12-alpha-Hydroxysteroid Dehydrogenase PP; Origin: Clostridium spec.
Jülich Enzyme Products — Lab

12-alpha-Hydroxysteroid Dehydrogenase; Origin: microorganisms
Asahi — Pilot

Glucose oxidase. — **1.1.3.4**
Glucose oxyhydrase. Beta-D-glucose:oxygen 1-oxido-reductase. Glucose aerodehydrogenase. D-Glucose-1-oxidase. — Beta-D-glucose + O(2) = D-glucono-1,5-lactone + H(2)O(2).

Glucose Oxidase
Seravac — Industrial

Glucose oxidase; Origin: Aspergillus niger
Amano: Hyderase — Industrial

Glucose oxidase; Origin: Aspergillus niger
Amano: Hyderase L — Industrial

Glucose oxidase; Origin: Aspergillus niger
Biozyme — Pilot

Glucose Oxidase; Origin: Aspergillus niger
Fluka — Industrial

Glucose oxidase; Origin: Aspergillus niger
Novozymes: Gluzyme® — Industrial

Glucose Oxidase; Origin: Aspergillus niger overproducer
Roche Diagnostics: Glucose Oxidase (GOD) — Industrial

Glucose Oxidase; Origin: Aspergillus sp.
Amano: Amano 2 [GO-2] — Pilot

Glucose Oxidase; Origin: Aspergillus sp.
Amano: Amano LC [GOLC] — Pilot

Glucose Oxidase; Origin: Aspergillus sp.
Amano: Amano LD2 [GOLD-2] — Pilot

Glucose Oxidase; Origin: Aspergillus sp.
Toyobo — Pilot

Glucose Oxidase; Origin: microorganism, rec. in yeast
Roche Diagnostics: Glucose Oxidase (GOD) — Pilot

Glucose Oxidase; Origin: Penicillium sp.
Biocatalysts — Industrial

Table 20.5. (cont.).

Cholesterol oxidase. **1.1.3.6**
Cholesterol-O2 oxidoreductase. Cholesterol + O(2) = cholest-4-en-3-one + H(2)O(2).

Cholesterol Oxidase
Asahi Pilot

Cholesterol Oxidase; Origin: Brevibacterium sterolicum, rec. in microorganism
Roche Diagnostics: Cholesterol Oxidase Pilot

Cholesterol Oxidase; Origin: microorganisms
Amano: Amano 6 [CHO-6] Pilot

Cholesterol Oxidase; Origin: microorganisms
Asahi Pilot

Cholesterol Oxidase; Origin: microorganisms
Toyobo Pilot

Cholesterol Oxidase; Origin: Nocardia erythropolis
Fluka Pilot

Cholesterol Oxidase; Origin: Pseudomonas sp.
Amano: Amano 1 [CHO-1] Pilot

Cholesterol Oxidase; Origin: Pseudomonas sp.
Amano: Amano 2 [CHO-2] Pilot

Cholesterol Oxidase; Origin: Pseudomonas sp.
Fluka Pilot

Cholesterol Oxidase; Origin: Streptomyces cinnamomeus
Asahi Pilot

Galactose oxidase. **1.1.3.9**
Beta-Galactose oxidase. D-galactose + O(2) = D-galacto-hexodialose + H(2)O(2).

Galactose Dehydrogenase; Origin: Agrobacterium sp.
Biocatalysts Pilot

Alcohol oxidase. **1.1.3.13**
Methanol oxidase. AOX. A primary alcohol + O(2) = an aldehyde + H(2)O(2).

Alcohol Oxidase; Origin: Candida sp.
Asahi Pilot

Alcohol oxidase, broad-range; Origin: microorganism, rec. in E. coli
BioCatalytics: BRAO-1001 Lab

Alcohol oxidase; Origin: Pichia pastoris
Biozyme Pilot

Alcohol Oxidase; Origin: Pichia pastoris
Jülich Enzyme Products Lab

Choline oxidase. **1.1.3.17**
Choline + O(2) = betaine aldehyde + H(2)O(2).

Choline Oxidase; Origin: Alcaligenes sp.
Fluka Pilot

Choline Oxidase; Origin: Arthrobacter globiformis
Asahi Pilot

Glycerol-3-phosphate oxidase. **1.1.3.21**
Sn-glycerol 3-phosphate + O(2) = glycerone phosphate + H(2)O(2).

L-Glycerophosphate Oxidase
Asahi Pilot

Glycerol 3-phosphate Oxidase; Origin: Aerococcus viridans
Fluka Lab

Table 20.5. (cont.).

L-Glycerophosphate Oxidase; Origin: Aerococcus viridans
Asahi — Pilot

L-Glycerol-3-phosphate Oxidase; Origin: microorganism, rec. in E. coli
Roche Diagnostics: L-Glycerol-3-phosphate Oxidase (GPO), stabilized — Pilot

L-alpha-Glycerophosphate Oxidase; Origin: microorganisms
Toyobo — Pilot

L-alpha-Glycerophosphate Oxidase; Origin: Pediococcus sp.
Toyobo — Pilot

L-alpha-Glycerophosphate Oxidase; Origin: Streptococcus sp.
Amano: Amano 2 [GPO-2] — Pilot

Xanthine oxidase. 1.1.3.22

Xanthine oxidoreductase. Hypoxanthine oxidase. Hypoxanthine-xanthine oxidase. Schardinger enzyme.

Xanthine + H(2)O + O(2) = urate + H(2)O(2).

Xanthine oxidase; Origin: buttermilk
Biozyme — Pilot

Xanthine Oxidase; Origin: buttermilk
Fluka — Pilot

Xanthine Oxidase; Origin: cow milk
Roche Diagnostics: Xanthine Oxidase — Pilot

Fructose 5-dehydrogenase. 1.1.99.11

D-Fructose dehydrogenase.

D-fructose + acceptor = 5-dehydro-D-fructose + reduced acceptor.

D-Fructose Dehydrogenase; Origin: Gluconobacter sp.
Toyobo — Pilot

Formate dehydrogenase. 1.2.1.2

Formate + NAD(+) = CO(2) + NADH.

Formate Dehydrogenase; Origin: Candida boidinii
Fluka — Pilot

Formate Dehydrogenase; Origin: Candida boidinii
Jülich Enzyme Products — Lab

Formate Dehydrogenase, rec.; Origin: Candida boidinii, overexpressed in E. coli
Roche Diagnostics: Formate Dehydrogenase (FDH), rec. — Industrial

Formate Dehydrogenase rec.; Origin: E. coli
Fluka — Lab

Formate Dehydrogenase; Origin: microorganisms
Unitika: Formate Dehydrogenase (FDH) — Pilot

Formate Dehydrogenase; Origin: Pseudomonas sp.
Fluka — Lab

Formate Dehydrogenase; Origin: Pseudomonas sp.
Fluka — Lab

Formate Dehydrogenase; Origin: Xilaria digitata (formerly Candida biodinii)
Roche Diagnostics: Formate Dehydrogenase (FDH) — Industrial

Formate Dehydrogenase; Origin: yeast
Fluka — Pilot

Aldehyde dehydrogenase (NAD(P)+). 1.2.1.5

An aldehyde + NAD(P)(+) + H(2)O = an acid + NAD(P)H.

Aldehyde Dehydrogenase; Origin: baker's yeast
Fluka — Lab

Table 20.5. (cont.).

Aldehyde dehydrogenase; Origin: yeast
Biozyme — Pilot

Aldehyde Dehydrogenase; Origin: yeast
Roche Diagnostics: Aldehyde Dehydrogenase (AldDH) — Lab

Glyceraldehyde 3-phosphate dehydrogenase (phosphorylating). **1.2.1.12**
NAD-dependent glyceraldehyde-3-phosphate dehydrogenase. Triosephosphate dehydrogenase. GAPDH.
D-glyceraldehyde 3-phosphate + phosphate + NAD(+) = 3-phospho-D-glyceroyl phosphate + NADH.

Glyceraldehyde-3-Phosphate Dehydrogenase (GapDH); Origin: Bacillus stearothermophilus
Unitika — Pilot

Glyceraldehyde-3-phosphate dehydrogenase; Origin: rabbit muscle
Biozyme — Pilot

Glyceraldehyde-3-phosphate Dehydrogenase; Origin: rabbit muscle
Fluka — Lab

Formaldehyde dehydrogenase. **1.2.1.46**
Formaldehyde + NAD(+) + H(2)O = formate + NADH.

Formaldehyde Dehydrogenase; Origin: Pseudomonas putida
Fluka — Lab

Formaldehyde Dehydrogenase; Origin: Pseudomonas sp.
Toyobo — Pilot

Pyruvate oxidase. **1.2.3.3**
Pyruvic oxidase.
Pyruvate + phosphate + O(2) + H(2)O = acetyl phosphate + CO(2) +H(2)O(2).

Pyruvate Oxidase ; Origin: Aerococcus viridans
Asahi — Pilot

Pyruvate Oxidase; Origin: Lactobacillus plantarum, rec. E. coli
Roche Diagnostics: Pyruvate Oxidase (PyrOD) — Pilot

Bilirubin oxidase. **1.3.3.5**
Bilirubin + O(2) = biliverdin + H(2)O.

Bilirubin Oxidase; Origin: Myrothecium sp.
Amano: Amano 2 [BO-2] — Pilot

Acyl-CoA oxidase. **1.3.3.6**
Acyl-CoA + O(2) = trans-2,3-dehydroacyl-CoA + H(2)O(2).

Acyl-CoA Oxidase; Origin: Arthrobacter sp.
Asahi — Pilot

Acyl-CoA Oxidase; Origin: microorganisms
Amano: Amano 3 [ACO-3] — Pilot

Alanine dehydrogenase. **1.4.1.1**
L-alanine + H(2)O + NAD(+) = pyruvate + NH(3) + NADH.

Alanine Dehydrogenase
Asahi — Pilot

L-Alanine Dehydrogenase; Origin: Bacillus cereus
Jülich Enzyme Products — Lab

Alanine Dehydrogenase; Origin: Bacillus stearothermophilus
Unitika: Alanine Dehydrogenase (AlaDH) — Pilot

L-Alanine Dehydrogenase; Origin: Bacillus subtilis
Fluka — Lab

Table 20.5. (cont.).

Glutamate dehydrogenase (NAD(P)+). **1.4.1.3**
Glutamic dehydrogenase.
L-glutamate + H(2)O + NAD(P)(+) = 2-oxoglutarate + NH(3) + NAD(P)H.

Glutamate dehydrogenase; Origin: beef liver
Biozyme — Pilot

Glutamate Dehydrogenase; Origin: bovine liver
Fluka — Pilot

L-Glutamate Dehydrogenase; Origin: bovine liver
Roche Diagnostics: L-Glutamate Dehydrogenase (GlDH), lyo. — Pilot

Glutamate Dehydrogenase; Origin: microorganisms
Toyobo — Pilot

Glutamate Dehydrogenase; Origin: Proteus sp.
Toyobo — Pilot

Leucine dehydrogenase. **1.4.1.9**
L-leucine + H(2)O + NAD(+) = 4-methyl-2-oxopentanoate + NH(3) + NADH.

Leucine Dehydrogenase; Origin: Bacillus cereus
Biocatalysts — Pilot

Leucine Dehydrogenase; Origin: Bacillus sp.
Toyobo — Pilot

Leucine Dehydrogenase; Origin: Bacillus stearothermophilus
Unitika: Leucine Dehydrogenase (LeuDH) — Pilot

Phenylalanine dehydrogenase. **1.4.1.20**
L-phenylalanine + H(2)O + NAD(+) = phenylpyruvate + NH(3) + NADH.

Phenylalanine Dehydrogenase; Origin: microorganisms
Unitika: Phenylalanine Dehydrogenase (PheDH) — Pilot

Phenylalanine Dehydrogenase; Origin: Sporosarcina sp.
Biocatalysts — Lab

D-amino acid oxidase. **1.4.3.3**
A D-amino acid + H(2)O + O(2) = a 2-oxo acid + NH(3) + H(2)O(2).

D-Amino Acid Oxidase; Origin: hog kidney
Fluka — Lab

D-Amino Acid Oxidase; Origin: hog kidney
Fluka — Lab

D-Amino Acid oxidase; Origin: porcine kidney
Biozyme — Pilot

D-Amino Acid Oxidase; Origin: Trigonopsis variabilis
Recordati: DAAO Beads — Industrial

D-Amino Acid Oxidase, carrier-fixed; Origin: Trigonopsis variabilis
Roche Diagnostics: D-Amino Acid Oxidase (D-AOD), carrier-fixed — Industrial

D-Amino acid Oxidase, immobilized; Origin: Trigonopsis variabilis
Fluka — Industrial

Amine oxidase (flavin-containing). **1.4.3.4**
Monoamine oxidase. Tyramine oxidase. Tyraminase. Amine oxidase.
RCH(2)NH(2) + H(2)O + O(2) = RCHO + NH(3) + H(2)O(2).

Tyramine Oxidase; Origin: Arthrobacter sp.
Asahi — Pilot

Table 20.5. (cont.).

Dihydrofolate reductase. **1.5.1.3**
Tetrahydrofolate dehydrogenase.
5,6,7,8-tetrahydrofolate + NADP(+) = 7,8-dihydrofolate + NADPH.

Dihydrofolate Reductase; Origin: bovine liver
Fluka — Lab

Sarcosine oxidase. **1.5.3.1**
Sarcosine + H(2)O + O(2) = glycine + formaldehyde + H(2)O(2).

Sarcosine Oxidase
Asahi — Pilot

Sarcosine Oxidase; Origin: microorganisms
Toyobo — Pilot

With other acceptors. **1.5.99.**

Dimethylamine Dehydrogenase; Origin: Paracoccus spec.
Jülich Enzyme Products — Lab

Trimethylamine dehydrogenase. **1.5.99.7**
TMADh.
Trimethylamine + H(2)O + acceptor = dimethylamine + formaldehyde +reduced acceptor.

Trimethylamine Dehydrogenase; Origin: Paracoccus spec.
Jülich Enzyme Products — Lab

Glutathione reductase (NADPH). **1.6.4.2**
NADPH + oxidized glutathione = NADP(+) + 2 glutathione.

Glutathione Reductase; Origin: baker's yeast
Fluka — Lab

NADPH dehydrogenase. **1.6.99.1**
NADPH diaphorase.
NADPH + acceptor = NADP(+) + reduced acceptor.

Diaphorase (NADPH); Origin: Bacillus megaterium
Asahi — Pilot

Diaphorase I; Origin: Bacillus stearothermophilus
Unitika — Pilot

Urate oxidase. **1.7.3.3**
Uricase.
Urate + O(2) + H(2)O = 5-hydroxyisourate + H(2)O(2).

Uricase; Origin: Arthrobacter globiformis
Asahi — Pilot

Uricase; Origin: Bacillus fastidiosus
Fluka — Lab

Uricase; Origin: Bacillus sp.
Toyobo — Pilot

Uricase; Origin: pig liver
Biozyme — Pilot

Dihydrolipoamide dehydrogenase. **1.8.1.4.**
Lipoamide reductase (NADH). E3 component of alpha-ketoacid dehydrogenase complexes. Lipoyl dehydrogenase. Dihydrolipoyl dehydrogenase.
Dihydrolipoamide + NAD(+) = lipoamide + NADH.

Diaphorase (NADH); Origin: Bacillus megaterium
Asahi — Pilot

Diaphorase II; Origin: Bacillus stearothermophilus
Unitika — Pilot

Table 20.5. (cont.).

Diaphorase; Origin: Clostridium kluyveri
Fluka — Pilot

Laccase. — **1.10.3.2**
Urishiol oxidase. — 4 benzenediol + O(2) = 4 benzosemiquinone + 2 H(2)O.

Laccase A; Origin: Agaricus bisporus
Jülich Enzyme Products — Lab

Laccase C; Origin: Coriolus versicolor
Jülich Enzyme Products — Lab

Laccase; Origin: rec. microorganism
Novozymes: DeniLite™ — Industrial

L-ascorbate oxidase. — **1.10.3.3**
Ascorbase. — 2 L-ascorbate + O(2) = 2 dehydroascorbate + 2 H(2)O.

Ascorbate Oxidase
Asahi — Pilot

Ascorbate Oxidase; Origin: Cucumber
Amano: Amano 2 [ASO-2] — Pilot

Ascorbate oxidase; Origin: Cucurbita sp.
Biozyme — Pilot

Ascorbate Oxidase; Origin: Cucurbity sp.
Fluka — Pilot

Ascorbate Oxidase; Origin: microorganisms
Amano: Amano 3 [ASO-3] — Pilot

Oxidoreductases. — **1.11.-.-**
Acting on a peroxide as acceptor (peroxidases).

Bromoperoxidase; Origin: Corallina officinalis
Fluka — Lab

Catalase. — **1.11.1.6**
2 H(2)O(2) = O(2) + 2 H(2)O.

Catalase
Biocatalysts: CATALASE — Industrial

Catalase
Seravac — Industrial

Catalase; Origin: Aspergillus niger
Amano: Catalase NL "Amano" — Industrial

Catalase; Origin: Aspergillus niger
Biozyme — Pilot

Catalase; Origin: Aspergillus niger
Fluka — Industrial

Catalase; Origin: Aspergillus niger
Novozymes: Catazyme® — Industrial

Catalase; Origin: Aspergillus niger
Roche Diagnostics: Catalase, technical grade — Industrial

Catalase; Origin: Aspergillus niger, rec.
Novozymes: Terminox™ Ultra — Industrial

Catalase; Origin: beef liver
Biozyme — Pilot

Catalase; Origin: beef liver
Roche Diagnostics — Industrial

Table 20.5. (cont.).

Catalase; Origin: bovine liver
Fluka — Industrial

Catalase, immobilized on Eupergit C; Origin: bovine liver
Fluka — Lab

Catalase; Origin: Corynebacterium glutamicum
Roche Diagnostics — Industrial

Catalase; Origin: Micrococcus lysodeikticus
Fluka — Lab

Catalase; Origin: microorganisms
Fluka — Pilot

Catalase; Origin: microorganisms
Toyobo — Industrial

Peroxidase. **1.11.1.7**
Myeloperoxidase. Donor + H(2)O(2) = oxidized donor + 2 H(2)O.

Lactoperoxidase; Origin: bovine milk
Biozyme — Pilot

Lactoperoxidase; Origin: bovine milk
Fluka — Pilot

Peroxidase; Origin: Coprinus cinereus
Novozymes: Novozym 502 — Industrial

Peroxidase; Origin: Coprinus cinereus
Novozymes: NS18010 — Industrial

Peroxidase; Origin: horse radish
Fluka — Industrial

Peroxidase; Origin: horseradish
Amano: Amano 2 [PO-2] — Pilot

Peroxidase; Origin: horseradish
Amano: Amano 3 [PO-3] — Pilot

Peroxidase; Origin: horseradish
Biocatalysts — Industrial

Peroxidase; Origin: horseradish
Biozyme — Pilot

Peroxidase; Origin: horseradish
Roche Diagnostics: Peroxidase (POD), Grade I — Industrial

Peroxidase; Origin: horseradish
Roche Diagnostics: Peroxidase (POD), Grade II — Pilot

Peroxidase ; Origin: horseradish
Seravac — Industrial

Peroxidase; Origin: horseradish
Toyobo — Pilot

Glutathione peroxidase. **1.11.1.9**
2 glutathione + H(2)O(2) = oxidized glutathione + 2 H(2)O.

Glutathione Peroxidase; Origin: bovine erythrocytes
Fluka — Lab

Chloride peroxidase. **1.11.1.10**
Chloroperoxidase. 2 RH + 2 chloride + H(2)O(2) = 2 RCl + 2 H(2)O.

Chloroperoxidase; Origin: Caldariomyces fumago
Fluka — Pilot

Table 20.5. (cont.).

Chloroperoxidase; Origin: Leptoxyphium fumago
Jülich Enzyme Products — Lab

Lipoxygenase. — **1.13.11.12**
Lipoxidase. Carotene oxidase. Lipoperoxidase.
Linoleate + O(2) = (9Z,11E)-(13S)-13-hydroperoxyoctadeca-9,11-dienoate.

Lipoxygenase II; Origin: pea, rec. in E. coli
Biocatalysts — Pilot

Lipoxygenase III; Origin: pea, rec. in E. coli
Biocatalysts — Pilot

Lipoxidase; Origin: soybean
Fluka — Lab

Lipoxidase; Origin: soybeen
Biozyme — Pilot

Lactate 2-monooxygenase. — **1.13.12.4**
Lactate oxidative decarboxylase. Lactate oxidase. Lactate oxygenase.
(S)-lactate + O(2) = acetate + CO(2) + H(2)O.

Lactate Oxidase
Asahi — Pilot

Lactate Oxidase; Origin: Pediococcus sp.
Fluka — Pilot

Oxidoreductases. — **1.14.13.-**
Acting on paired donors with incorporation of molecular oxygen.
With NADH or NADPH as one donor, and incorporation of one atom of oxygen.

2-Tridecanone Monooxygenase; Origin: Pseudomonas cepacia
Fluka — Lab

Cyclopentanone monooxygenase. — **1.14.13.16**
Cyclopentanone + NADPH + O(2) = 5-valerolactone + NADP(+) + H(2)O.

Cyclopentanone Monooxygenase; Origin: Pseudomonas sp.
Fluka — Lab

Cyclohexanone monooxygenase. — **1.14.13.22**
Cyclohexanone oxygenase.
Cyclohexanone + NADPH + O(2) = 6-hexanolide + NADP(+) + H(2)O.

Cyclohexanone Monooxygenase; Origin: Acinetobacter sp.
Fluka — Lab

Cyclohexanone Monooxygenase; Origin: E. coli overproducer
Fluka — Lab

Cyclohexanone Monooxygenase; Origin: Nocardia globerula
Fluka — Lab

Cyclohexanone Monooxygenase; Origin: Xanthobacter sp.
Fluka — Lab

2-hydroxybiphenyl 3-monooxygenase. — **1.14.13.44**
2-hydroxybiphenyl + NADH + O(2) = 2,3-dihydroxybiphenyl + NAD(+) + H(2)O.

2-Hydroxybiphenylmonooxygenase; Origin: E. coli
Fluka — Lab

Table 20.5. (cont.).

Camphor 5-monooxygenase. **1.14.15.1**
Camphor 5-exo-methylene hydroxylase. Cytochrome p450-cam. (+)-camphor + putidaredoxin + O(2) = (+)-exo-5-hydroxycamphor + oxidizedputidaredoxin + H(2)O.

(+)-Camphor Monooxygenase; Origin: Pseudomonas putida
Fluka — Lab

Monophenol monooxygenase. **1.14.18.1**
Tyrosinase. Phenolase. Monophenol oxidase. Cresolase. L-tyrosine + L-DOPA + O(2) = L-DOPA + DOPAquinone + H(2)O.

Tyrosinase; Origin: mushroom
Fluka — Lab

Progesterone monooxygenase. **1.14.99.4**
Progesterone hydroxylase. Progesterone + AH(2) + O(2) = testosterone acetate + A + H(2)O.

Progesterone Monooxygenase; Origin: Cylindrocapron radicicola
Fluka — Lab

Superoxide dismutase. **1.15.1.1**
2 peroxide radical + 2 H(+) = O(2) + H(2)O(2).

Superoxide Dismutase; Origin: Bacillus stearothermophilus
Unitika: Superoxide Dismutase (SOD) — Pilot

Superoxide dismutase; Origin: bovine erythrocytes
Biozyme — Pilot

Superoxide Dismutase; Origin: bovine erythrocytes
Fluka — Pilot

Superoxide Dismutase; Origin: bovine erythrocytes
Roche Diagnostics: Superoxide Dismutase (SOD) — Lab

Superoxide Dismutase; Origin: bovine liver
Fluka — Pilot

Catechol O-methyltransferase. **2.1.1.6**
S-adenosyl-L-methionine + catechol = S-adenosyl-L-homocysteine +guaiacol.

Brenzkatechin-O-methyl-Transferase
Fluka — Lab

Transketolase. **2.2.1.1**
Glycoaldehyde transferase. Sedoheptulose 7-phosphate + D-glyceraldehyde 3-phosphate = D-ribose 5-phosphate + D-xylulose 5-phosphate.

Transketolase; Origin: baker's yeast
Fluka — Lab

Transketolase ; Origin: E. coli
Fluka — Lab

Transketolase; Origin: E. coli K12 (rec.)
Jülich Enzyme Products — Lab

Transaldolase. **2.2.1.2**
Dihydroxyacetone transferase. Glycerone transferase. Sedoheptulose 7-phosphate + D-glyceraldehyde 3-phosphate = D-erythrose 4-phosphate + D-fructose 6-phosphate.

Transaldolase ; Origin: Candida utilis
Fluka — Lab

Transaldolase; Origin: E. coli K12 (rec.)
Jülich Enzyme Products — Lab

Table 20.5. (cont.).

Glucosamine-phosphate N-acetyltransferase.	**2.3.1.4**
Phosphoglucosamine transacetylase. Phosphoglucosamine acetylase.	Acetyl-CoA + D-glucosamine 6-phosphate = CoA + N-acetyl-D-glucosamine 6-phosphate.
Phosphotransacetylase; Origin: Bacillus stearothermophilus	
Unitika: Phosphotransacetylase (PTA)	Lab
Carnitine O-acetyltransferase.	**2.3.1.7**
Carnitine acetylase.	Acetyl-CoA + carnitine = CoA + O-acetylcarnitine.
Carnitine Acetyltransferase; Origin: pigeon breast muscle	
Fluka	Lab
Gamma-glutamyltransferase.	**2.3.2.2**
Gamma-glutamyltranspeptidase. Glutamyl transpeptidase.	(5-L-glutamyl)-peptide + an amino acid = peptide + 5-L-glutamyl-aminoacid.
gamma-Glutamyltransferase; Origin: beef kidney	
Biozyme	Pilot
gamma-Glutamyl Transpeptidase; Origin: hog kidney	
Fluka	Lab
Phosphorylase.	**2.4.1.1**
Muscle phosphorylase A and B. Amylophosphorylase. Polyphosphorylase.	{(1,4)-alpha-D-glucosyl}(N) + phosphate = {(1,4)-alpha-D-glucosyl}(N-1)+ alpha-D-glucose 1-phosphate.
Phosphorylase b; Origin: rabbit muscle	
Fluka	Lab
Sucrose synthase.	**2.4.1.13**
UDP-glucose-fructose glucosyltransferase. Sucrose-UDP glucosyltransferase.	UDP-glucose + D-fructose = UDP + sucrose.
Sucrose Synthase; Origin: rice grains	
Jülich Enzyme Products	Lab
1,4-alpha-glucan branching enzyme.	**2.4.1.18**
Glycogen branching enzyme. Amylo-(1,4 to 1,6)transglucosidase. Branching enzyme. Amylo-(1,4-1,6)-transglycosylase.	Formation of 1,6-glucosidic linkages of glycogen.
Transglucosidase; Origin: Aspergillus niger	
Amano: Transglucosidase L "Amano"	Industrial
Lactose synthase.	**2.4.1.22**
UDP-galactose-glucose galactosyltransferase. N-acetyllactosamine synthase.	UDP-galactose + D-glucose = UDP + lactose.
beta-1,4-Galactosyl Transferase; Origin: bovine milk	
Fluka	Pilot
Beta-N-acetylglucosaminyl-glycopeptide beta-1,4-galactosyltransferase.	**2.4.1.38**
Glycoprotein 4-beta-galactosyltransferase. Thyroid galactosyltransferase. UDP-galactose-glycoprotein galactosyltransferase.	UDP-galactose + N-acetyl-beta-D-glucosaminylglycopeptide = UDP +beta-D-galactosyl-1,4-N-acetyl-beta-D-glucosaminylglycopeptide.
Beta-1,4-Galactosyltransferase; Origin: Saccharomyces cerevisiae (rec.)	
Jülich Enzyme Products	Lab

Table 20.5. (cont.).

N-acetyllactosaminide alpha-1,3-galactosyltransferase. **2.4.1.151**

Galactosyltransferase.

UDP-galactose + beta-D-galactosyl-(1,4)-N-acetyl-D-glucosaminyl-R = UDP + alpha-D-galactosyl-(1,3)-beta-D-galactosyl-(1,4)-N-acetyl-D-glucos

alpha-1,3-Galactosyl-Transferase; Origin: E. coli, rec.
Fluka — Lab

alpha-1,3-Galactosyltransferase; Origin: E. coli, rec.
Fluka — Lab

Purine-nucleoside phosphorylase. **2.4.2.1**

Inosine phosphorylase. PNPase.

Purine nucleoside + phosphate = purine + alpha-D-ribose1-phosphate.

Purine-Nucleoside phosphorylase
Asahi — Pilot

Purine-Nucleoside Phosphorylase; Origin: microorganisms
Toyobo — Pilot

Transferases. **2.6.1.-**
Transferring nitrogenous groups.
Transaminases (aminotransferases).

Transaminase, branched-chain, L-specific; Origin: microorganism, rec. in E. coli
BioCatalytics: AT-102 — Lab

Transaminase, broad-range, D-specific; Origin: microorganism, rec. in E. coli
BioCatalytics: AT-103 — Lab

Transaminase, broad-range, L-specific; Origin: microorganism, rec. in E. coli
BioCatalytics: AT-101 — Lab

Aspartate aminotransferase. **2.6.1.1**

Transaminase A. Glutamic--oxaloacetic transaminase. Glutamic--aspartic transaminase.

L-aspartate + 2-oxoglutarate = oxaloacetate + L-glutamate.

Glutamic-oxaloacetic transaminase; Origin: pig heart
Biozyme — Pilot

Glutamate-Oxaloacetate Transaminase; Origin: pig heart (mitochondrial)
Roche Diagnostics: Glutamate-Oxaloacetate Transaminase (GOT) — Pilot

Glutamic-Oxalacetic Transaminase; Origin: porcine heart
Fluka — Pilot

Alanine aminotransferase. **2.6.1.2**

Glutamic--pyruvic transaminase. Glutamic--alanine transaminase.

L-alanine + 2-oxoglutarate = pyruvate + L-glutamate.

Glutamate-Pyruvate Transaminase; Origin: pig heart
Roche Diagnostics: Glutamate-Pyruvate Transaminase (GPT) — Pilot

Glutamic-pyruvic transaminase; Origin: pig heart
Biozyme — Pilot

Glutamic-Pyruvic Transaminase; Origin: porcine heart
Fluka — Pilot

Hexokinase. **2.7.1.1**

Glucokinase. Hexokinase type IV.

ATP + D-hexose = ADP + D-hexose 6-phosphate.

Hexokinase
Asahi — Pilot

Hesperidinase; Origin: Penicillium decumbens
Amano: Hesperidinase "Amano" Conc. — Industrial

Table 20.5. (cont.).

Hexokinase; Origin: Saccharomyces sp. Toyobo	Pilot
Hexokinase; Origin: yeast Biozyme	Pilot
Hexokinase; Origin: yeast Fluka	Industrial
Hexokinase; Origin: yeast Fluka	Industrial
Glucokinase.	**2.7.1.2**
Glucose kinase.	ATP + D-glucose = ADP + D-glucose 6-phosphate.
Glucokinase; Origin: Bacillus stearothermophilus Unitika: Glucokinase (GlcK)	Pilot
Glucokinase; Origin: Zymomonas mobilis Unitika: Glucokinase (GlcK)	Pilot
6-phosphofructokinase.	**2.7.1.11**
Phosphohexokinase. Phosphofructokinase I.	ATP + D-fructose 6-phosphate = ADP + D-fructose 1,6-bisphosphate.
Phosphofructokinase; Origin: Bacillus stearothermophilus Unitika: Phosphofructokinase (PFK)	Pilot
Glycerol kinase.	**2.7.1.30**
Glycerokinase. ATP:glycerol 3-phosphotransferase.	ATP + glycerol = ADP + glycerol 3-phosphate.
Glycerol Kinase Asahi	Pilot
Glycerol Kinase; Origin: Arthrobacter sp. Amano: Amano 2 [GK-2]	Pilot
Glycerokinase; Origin: Bacillus stearothermophilus Roche Diagnostics: Glycerokinase, lyo.	Pilot
Glycerokinase; Origin: Bacillus stearothermophilus Roche Diagnostics: Glycerokinase, sol.	Pilot
Glycerol Kinase; Origin: E. coli Fluka	Pilot
Glycerol Kinase; Origin: microorganisms Toyobo	Pilot
Pyruvate kinase.	**2.7.1.40**
Phosphoenolpyruvate kinase. Phosphoenol transphosphorylase.	ATP + pyruvate = ADP + phosphoenolpyruvate.
Pyruvate Kinase; Origin: Bacillus stearothermophilus Unitika: Pyruvate Kinase (PK)	Pilot
Pyruvate Kinase; Origin: pig heart Biozyme	Pilot
Pyruvate Kinase; Origin: rabbit muscle Biozyme	Pilot
Pyruvate Kinase; Origin: rabbit muscle Fluka	Lab
Pyruvate Kinase; Origin: Zymomonas mobilis Unitika: Pyruvate Kinase (PK)	Pilot

Table 20.5. (cont.).

Streptomycin 6-kinase. **2.7.1.72**
Streptidine kinase. Streptomycin 6-phosphotransferase. APH(6). ATP + streptomycin = ADP + streptomycin 6-phosphate.

Streptokinase; Origin: Streptococcus hemolyticus
Fluka — Lab

Acetate kinase. **2.7.2.1**
Acetokinase. ATP + acetate = ADP + acetyl phosphate.

Acetate Kinase; Origin: Bacillus stearothermophilus
Unitika: Acetate Kinase (AK) — Pilot

Acetate Kinase; Origin: E. coli
Fluka — Lab

Phosphoglycerate kinase. **2.7.2.3**
ATP + 3-phospho-D-glycerate = ADP + 3-phospho-D-glyceroyl phosphate.

Phosphoglycerate Kinase; Origin: Bacillus stearothermophilus
Unitika: Phosphoglycerate Kinase (PGK) — Pilot

Creatine kinase. **2.7.3.2**
ATP + creatine = ADP + phosphocreatine.

Creatine Kinase; Origin: beef heart
Biozyme — Pilot

Creatine Kinase; Origin: pig heart
Biozyme — Pilot

Creatine Kinase; Origin: rabbit muscle
Biozyme — Pilot

Creatine Phosphokinase; Origin: rabbit muscle
Fluka — Lab

Adenylate kinase. **2.7.4.3**
Myokinase. Adenylic kinase. Adenylokinase. ATP + AMP = ADP + ADP.

Adenylate Kinase; Origin: Bacillus stearothermophilus
Unitika: Adenylate Kinase (AdK) — Pilot

Polyribonucleotide nucleotidyltransferase. **2.7.7.8**
Polynucleotide phosphorylase. {RNA}(N+1) + phosphate = {RNA}(N) + a nucleoside diphosphate.

Polynucleotide Phosphorylase; Origin: Bacillus stearothermophilus
Unitika: Polynucleotide Phosphorylase (PNPase) — Pilot

Acylneuraminate cytidylyltransferase. **2.7.7.43**
CMP-N-acetylneuraminic acid synthetase. CMP-NeuNAc synthetase. CMP-sialate pyrophosphorylase. CMP-sialate diphosphorylase. CTP + N-acylneuraminate = diphosphate + CMP-N-acylneuraminate.

CMP-Neu5Ac Synthetase; Origin: E. coli K 235/CS1
Jülich Enzyme Products — Lab

Hydrolases. **3.1.1.-**
Acting on ester bonds.
Carboxylic ester hydrolases.

Lipases & Esterases Screening Set; Origin: mammalian sources and microorganisms
Roche Diagnostics: CHIRAZYME Lipases & Esterases, Screening Set Industrial Enzymes 2 — Industrial

Table 20.5. (cont.).

Carboxylesterase. **3.1.1.1**

Ali-esterase. B-esterase. Monobutyrase. Cocaine esterase. A carboxylic ester + H(2)O = an alcohol + a carboxylic anion.

Esterase basic kit Fluka	Lab
Esterase; Origin: Bacillus sp. Fluka	Lab
Esterase; Origin: Bacillus stearothermophilus Fluka	Lab
Esterase; Origin: Bacillus thermoglucosidasius Fluka	Lab
Esterase; Origin: Candida lipolytica Fluka	Lab
Esterase ; Origin: Candida rugosa Altus	Industrial
Esterase; Origin: hog liver Fluka	Industrial
Esterase, immobilized on Eupergit® C; Origin: hog liver Fluka	Pilot
Esterase Isoenzyme 1; Origin: hog liver Fluka	Pilot
Esterase; Origin: horse liver Fluka	Lab
Esterase Screening Kit ; Origin: microorganism, rec. in E. coli ThemoGen: QuickScreen Esterase Kits	Lab
Esterase; Origin: Mucor miehei Fluka	Lab
Esterase; Origin: pig liver Jülich Enzyme Products: Esterase PL	Lab
Esterase. immobilized; Origin: pig liver Roche Diagnostics: CHIRAZYME E-1, c.-f., lyo.	Industrial
Pig Liver Esterase; Origin: pig Liver Altus	Industrial
Pig Liver Esterase; Origin: pig liver Roche Diagnostics: PLE, technical grade, susp.	Industrial
Esterase; Origin: pig liver, fraction 1 Roche Diagnostics: CHIRAZYME E-1, lyo.	Industrial
Esterase; Origin: pig liver, fraction 2 Roche Diagnostics: CHIRAZYME E-2, lyo.	Industrial
Esterase; Origin: Rhizopus arrhizus Jülich Enzyme Products: Esterase EL9	Lab
Esterase; Origin: Rhodotorula pilimanae Jülich Enzyme Products: Esterase EL5	Lab
Esterase; Origin: Saccharomyces cerevisiae Fluka	Lab
Desacetyl-esterase; Origin: Therm.sp., rec. in E. coli. Recordati: Desa-REC	Industrial
Esterase; Origin: Thermoanaerobium brockii Fluka	Lab

Table 20.5. (cont.).

Triacylglycerol lipase. **3.1.1.3**

Lipase. Triglyceride lipase. Tributyrase.

Triacylglycerol + H(2)O = diacylglycerol + a fatty acid anion.

Lipase basic kit Fluka	Lab
Lipase extension kit Fluka	Lab
Monoglyceride Lipase Asahi	Pilot
Lipase; Origin: Achromobacter sp. Meito Sangyo: Lipase AL	Industrial
Lipase, immobilized; Origin: Achromobacter sp. Meito Sangyo: Lipase ALC/ALG	Industrial
Lipase; Origin: Alcaligenes sp. Altus	Industrial
Lipase; Origin: Alcaligenes sp. Meito Sangyo: Lipase PL	Industrial
Lipase; Origin: Alcaligenes sp. Meito Sangyo: Lipase QLL	Industrial
Lipase; Origin: Alcaligenes sp. Meito Sangyo: Lipase QLM	Industrial
Lipase, immobilized; Origin: Alcaligenes sp. Meito Sangyo: Lipase PLC/PLG	Industrial
Lipase, immobilized; Origin: Alcaligenes sp. Meito Sangyo: Lipase QLC/QLG	Industrial
Lipase; Origin: Alcaligines sp. Roche Diagnostics: CHIRAZYME L-10, lyo.	Industrial
Lipase; Origin: Aspergillus niger Altus	Industrial
Lipase; Origin: Aspergillus niger Amano: Lipase A "Amano" 6	Industrial
Lipase; Origin: Aspergillus niger Amano: Lipase AS	Industrial
Lipase; Origin: Aspergillus niger Amano: Lipase DS	Industrial
Lipase; Origin: Aspergillus niger Fluka	Industrial
Lipase; Origin: Aspergillus niger Fluka	Industrial
Lipase, immobilized in Sol-Gel-AK; Origin: Aspergillus niger Fluka	Lab
Lipase; Origin: Aspergillus oryzae Fluka	Industrial
Lipase; Origin: Burkholderia cepacia Meito Sangyo: Lipase SL	Industrial
Lipase ; Origin: Burkholderia sp. Fluka	Pilot
Lipase; Origin: Candida antarctica Fluka	Lab
Lipase A; Origin: Candida antarctica Fluka	Industrial

Table 20.5. (cont.).

Lipase, immobilized; Origin: Candida antarctica Fluka	Industrial
Lipase, immobilized in Sol-Gel-AK; Origin: Candida antarctica Fluka	Pilot
Lipase, immobilized in Sol-Gel-AK on sintered glass; Origin: Candida antarctica Fluka	Lab
Lipase, type A; Origin: Candida antarctica Altus	Industrial
Lipase, type B; Origin: Candida antarctica Altus	Industrial
Lipase B; Origin: Candida antarctica, rec. Fluka	Industrial
Lipase; Origin: Candida antarctica, type A Roche Diagnostics: CHIRAZYME L-5, lyo.	Industrial
Lipase; Origin: Candida antarctica, type A Roche Diagnostics: CHIRAZYME L-5, sol.	Industrial
Lipase, immobilized; Origin: Candida antarctica, type A Roche Diagnostics: CHIRAZYME L-5, c.-f., lyo.	Industrial
Lipase; Origin: Candida antarctica, type A, rec. in Aspergillus oryzae Novozymes: NovoCor AD	Industrial
Lipase; Origin: Candida antarctica, type A, rec. in Aspergillus oryzae Novozymes: Novozym® 868	Industrial
Lipase; Origin: Candida antarctica, type B Roche Diagnostics: CHIRAZYME L-2, lyo.	Industrial
Lipase; Origin: Candida antarctica, type B Roche Diagnostics: CHIRAZYME L-2, sol.	Industrial
Lipase, immobilized; Origin: Candida antarctica, type B Roche Diagnostics: CHIRAZYME L-2, c.-f., C2, lyo. (Novozym 435)	Industrial
Lipase, immobilized; Origin: Candida antarctica, type B Roche Diagnostics: CHIRAZYME L-2, c.-f., C3, lyo.	Industrial
Lipase, immobilized; Origin: Candida antarctica, type B Roche Diagnostics: CHIRAZYME L-2, c.-f., lyo.	Industrial
Lipase; Origin: Candida antarctica, type B, rec. in Aspergillus oryzae Novozymes: Nocozym 525 L	Industrial
Lipase, immobilized; Origin: Candida antarctica, type B, rec. in Aspergillus oryzae Novozymes: Novozym® 435	Industrial
Lipase; Origin: Candida cylindracae Jülich Enzyme Products: Lipase LE11	Lab
Lipase; Origin: Candida cylindracea Biocatalysts	Industrial
Lipase; Origin: Candida cylindracea Fluka	Industrial
Lipase; Origin: Candida cylindracea Fluka	Industrial
Lipase; Origin: Candida cylindracea Meito Sangyo: Lipase MY	Industrial
Lipase; Origin: Candida cylindracea Meito Sangyo: Lipase OF	Industrial
Lipase; Origin: Candida cylindracea Meito Sangyo: Lipase OFL	Industrial

Table 20.5. (cont.).

Lipase, immobilized; Origin: Candida cylindracea Meito Sangyo: Lipase OFC/OFG	Industrial
Lipase, immobilized in Sol-Gel-AK; Origin: Candida cylindracea Fluka	Pilot
Lipase; Origin: Candida lipolytica Fluka	Pilot
Lipase; Origin: Candida lypolytica Altus	Industrial
Lipase; Origin: Candida rugosa Altus	Industrial
Lipase; Origin: Candida rugosa Altus: ChiroCLEC-CR (dry)	Industrial
Lipase; Origin: Candida rugosa Altus: ChiroCLEC-CR (slurry)	Industrial
Lipase; Origin: Candida rugosa Amano: Lipase AY "Amano" 30	Industrial
Lipase; Origin: Candida rugosa Amano: Lipase AYS	Industrial
Lipase; Origin: Candida rugosa (formerly C. cylindracea) Roche Diagnostics: CHIRAZYME L-3, lyo.	Industrial
Lipase, purified; Origin: Candida rugosa (formerly C. cylindracea) Roche Diagnostics: CHIRAZYME L-3, purified, lyo.	Pilot
Lipase, purified, immobilized; Origin: Candida rugosa (formerly C. cylindracea) Roche Diagnostics: CHIRAZYME L-3, purified, c.-f., C2, lyo.	Pilot
Lipase; Origin: Candida utilis Fluka	Lab
Lipase; Origin: Chromobacterium viscosum Altus	Industrial
Lipase; Origin: Cromobacterium viscosum Asahi	Pilot
Lipase; Origin: Geotrichum candidum Altus	Industrial
Lipase; Origin: hog pancreas Fluka	Lab
Lipase; Origin: hog pancreas Fluka	Industrial
Lipase, immobilized in Sol-Gel-AK; Origin: hog pancreas Fluka	Pilot
Pancreatin; Origin: hog pancreas Fluka	Industrial
Lipase; Origin: Humicola sp. Roche Diagnostics: CHIRAZYME L-8, sol.	Industrial
Lipase B, covalently linked to carrier; Origin: microorganisms Fluka	Pilot
Lipoprotein Lipase; Origin: microorganisms Amano: Amano 6 [LPL-6]	Pilot
Lipase; Origin: Mucor javanicus Altus	Industrial
Lipase; Origin: Mucor javanicus Amano: Lipase M "Amano" 10	Industrial

Table 20.5. (cont.).

Lipase; Origin: Mucor javanicus Fluka	Industrial
Lipase; Origin: Mucor meihei Altus	Industrial
Lipase; Origin: Mucor miehei Fluka	Lab
Lipase; Origin: Mucor miehei Roche Diagnostics: CHIRAZYME L-9, lyo.	Industrial
Lipase; Origin: Mucor miehei Roche Diagnostics: CHIRAZYME L-9, sol.	Industrial
Lipase, immobilized; Origin: Mucor miehei Fluka: Lipozyme®, immobilized	Industrial
Lipase, immobilized; Origin: Mucor miehei Roche Diagnostics: CHIRAZYME L-9, c.-f., C2, lyo.	Industrial
Lipase, immobilized; Origin: Mucor miehei Roche Diagnostics: CHIRAZYME L-9, c.-f., dry	Industrial
Lipase, immobilized in Sol-Gel-AK; Origin: Mucor miehei Fluka	Lab
Lipase, immobilized in Sol-Gel-AK on sintered glass; Origin: Mucor miehei Fluka	Lab
Lipase; Origin: Mucor miehei, rec. Fluka	Industrial
Lipase; Origin: Penicillium camembertii Amano: Lipase G "Amano" 50	Industrial
Lipase; Origin: Penicillium roqueforti Fluka	Pilot
Lipase; Origin: Penicillium roquefortii Amano: Lipase R	Industrial
Lipase; Origin: porcine pancreas Altus	Industrial
Lipase; Origin: porcine pancreas Roche Diagnostics: CHIRAZYME L-7, lyo.	Industrial
Lipase; Origin: porcine pancreas Roche Diagnostics: Lipase	Pilot
Lipase; Origin: Protein engineered in rec. Aspergillus Novozymes: Lipolase Ultra	Industrial
Lipase; Origin: Protein engineered in rec. Aspergillus Novozymes: LipoPrime™	Industrial
Lipase; Origin: Pseudomonas aeroginosa Altus	Industrial
Lipase; Origin: Pseudomonas cepacia Altus	Industrial
Lipase; Origin: Pseudomonas cepacia Altus: ChiroCLEC-PC (dry)	Industrial
Lipase; Origin: Pseudomonas cepacia Altus: ChiroCLEC-PC (slurry)	Industrial
Lipase; Origin: Pseudomonas cepacia Amano: Lipase PS	Industrial
Lipase; Origin: Pseudomonas cepacia Amano: Lipase PS-C	Industrial

Table 20.5. (cont.).

Lipase; Origin: Pseudomonas cepacia Amano: Lipase PS-D	Industrial
Lipase; Origin: Pseudomonas cepacia Fluka	Industrial
Lipase; Origin: Pseudomonas cepacia Fluka	Industrial
Lipase, immobilized in Sol-Gel-AK; Origin: Pseudomonas cepacia Fluka	Pilot
Lipase, immobilized in Sol-Gel-AK on sintered glass; Origin: Pseudomonas cepacia Fluka	Lab
Lipase, immobilized on Ceramic particles; Origin: Pseudomonas cepacia Fluka	Lab
Lipase; Origin: Pseudomonas fluorescens Amano: Lipase AK	Industrial
Lipase; Origin: Pseudomonas fluorescens Fluka	Pilot
Lipase, immobilized in Sol-Gel-AK; Origin: Pseudomonas fluorescens Fluka	Pilot
Lipase, immobilized in Sol-Gel-AK on sintered glass; Origin: Pseudomonas fluorescens Fluka	Lab
Lipase, immobilized on Eupergit C; Origin: Pseudomonas fluorescens Fluka	Lab
Lipase; Origin: Pseudomonas sp. Roche Diagnostics: CHIRAZYME L-6, lyo.	Industrial
Lipase, immobilized; Origin: Pseudomonas sp. Toyobo	Industrial
Lipoprotein Lipase; Origin: Pseudomonas sp. Amano: Amano 3 [LPL-3]	Pilot
Lipoprotein Lipase; Origin: Pseudomonas sp. Toyobo	Pilot
Lipase; Origin: Pseudomonas stutzeri Meito Sangyo: Lipase TL	Industrial
Lipase; Origin: Rhizomucor miehei DSM Gist-brocades: Piccantase	Industrial
Lipase; Origin: Rhizomucor miehei Fluka	Industrial
Lipase; Origin: Rhizomucor miehei, rec. in Aspergillus oryzae Novozymes: Novozym® 388	Industrial
Lipase; Origin: Rhizomucor miehei, rec. in Aspergillus oryzae Novozymes: Palatase®	Industrial
Lipase, immobilized; Origin: Rhizomucor miehei, rec. in Aspergillus oryzae Novozymes: Lipozyme® RM IM	Industrial
Lipase; Origin: Rhizopus arrhizus Fluka	Industrial
Lipase; Origin: Rhizopus delemar Altus	Industrial
Lipase; Origin: Rhizopus delemar Fluka	Industrial
Lipase; Origin: Rhizopus niveus Altus	Industrial

Table 20.5. (cont.).

Lipase; Origin: Rhizopus niveus Amano: Newlase F	Industrial
Lipase; Origin: Rhizopus niveus Fluka	Pilot
Lipase; Origin: Rhizopus niveus Jülich Enzyme Products: Lipase LE9	Lab
Lipase; Origin: Rhizopus oryzae Altus	Industrial
Lipase; Origin: Rhizopus oryzae Amano: Lipase F-AP15	Industrial
Lipase; Origin: Rhizopus oryzae Amano: Lipase F-DS	Industrial
Lipase; Origin: Rhizopus sp. Meito Sangyo: Lipase UL	
Lipase; Origin: Thermomyces lanuginosa Altus	Industrial
Lipase; Origin: Thermomyces lanuginosa, rec. Aspergillus oryzae Novozymes: Novozym 677 BG	Industrial
Lipase; Origin: Thermomyces lanuginosa rec. in Aspergillus oryzae Novozymes: Greasex®	Industrial
Lipase; Origin: Thermomyces lanuginosa rec. in Aspergillus oryzae Novozymes: Lipolase®	Industrial
Lipase; Origin: Thermomyces lanuginosa rec. in Aspergillus oryzae Novozymes: Novozym® 27007	Industrial
Lipase; Origin: Thermomyces lanuginosa rec. in Aspergillus oryzae Novozymes: Novozym® 398	Industrial
Lipase; Origin: Thermomyces lanuginosa rec. in Aspergillus oryzae Novozymes: Novozym® 735	Industrial
Lipase; Origin: Thermomyces lanuginosa rec. in Aspergillus oryzae Novozymes: Novozym® 871	Industrial
Lipase; Origin: Thermomyces sp. (formerly Humicola sp.) Roche Diagnostics: CHIRAZYME L-8, lyo.	Industrial
Lipase; Origin: Thermus aquaticus Fluka	Lab
Lipase; Origin: Thermus flavus Fluka	Lab
Lipase; Origin: Thermus thermophilus Fluka	Lab
Lipase; Origin: wheat germ Fluka	Pilot
Phospholipase A2. Phosphatidylcholine 2-acylhydrolase. Lecithinase A. Phosphatidase. Phosphatidolipase.	**3.1.1.4** Phosphatidylcholine + H(2)O = 1-acylglycerophosphocholine + a fattyacid anion.
Phospholipase A2; Origin: Agkistrodon halys Fluka	Lab
Phospholipase A2; Origin: bovine pancreas Fluka	Pilot
Phospholipase A2; Origin: hog pancreas Fluka	Pilot

Table 20.5. (cont.).

Phospholipase A2; Origin: porcine pancreas
Biocatalysts — Pilot

Phospholipase A2; Origin: porcine pancreas
Novozymes: Lecitase® — Industrial

Phospholipase A2; Origin: Streptomyces violaceoruber
Asahi — Pilot

Acetylcholinesterase. **3.1.1.7**
True cholinesterase. Choline esterase I. Cholinesterase.
Acetylcholine + H(2)O = choline + acetate.

Acetylcholinesterase; Origin: bovine erythocytes
Biozyme — Pilot

Acetylcholine Esterase; Origin: Electrophorus electricus
Fluka — Lab

Cholinesterase. **3.1.1.8**
Pseudocholinesterase. Acylcholine acylhydrolase. Butyrylcholine esterase. Non-specific cholinesterase.
An acylcholine + H(2)O = choline + a carboxylic acid anion.

Butyrylcholine esterase; Origin: horse serum
Biozyme — Pilot

Butyrylcholine Esterase; Origin: horse serum
Fluka — Lab

Pectinesterase. **3.1.1.11**
Pectin methylesterase. Pectin demethoxylase. Pectin methoxylase.
Pectin + N H(2)O = N methanol + pectate.

Pectinesterase
Novozymes: Cellubrix® — Industrial

Pectinesterase
Novozymes: Novoclair™ FCE — Industrial

Pectinesterase
Novozymes: Pectinex BE — Industrial

Pectinesterase; Origin: Aspergillus niger
Amano: Pectinase P — Industrial

Pectinesterase; Origin: Aspergillus niger
Amano: Pectinase PL "Amano" — Industrial

Pectin Esterase; Origin: orange peel
Fluka — Industrial

Pectinesterase; Origin: rec. microorganism
Novozymes: Novoshape® — Industrial

Pectinesterase; Origin: rec. microorganism
Novozymes: Pectinex SMASH — Industrial

Sterol esterase. **3.1.1.13**
Cholesterol esterase. Cholesterol ester synthase. Triterpenol esterase.
A steryl ester + H(2)O = a sterol + a fatty acid.

Cholesterol Esterase
Asahi — Pilot

Cholesterol Esterase, lyo.; Origin: Candida rugosa (formerly C. cylindracea)
Roche Diagnostics: Cholesterol Esterase, lyo. — Pilot

Cholesterol Esterase, sol.; Origin: Candida rugosa (formerly C. cylindracea)
Roche Diagnostics: Cholesterol Esterase, sol. — Pilot

Cholesterol Esterase; Origin: hog pancreas
Fluka — Pilot

Table 20.5. (cont.).

Cholesterol esterase; Origin: pig pancreas
Biozyme — Pilot

Cholesterol Esterase; Origin: Pseudomonas sp.
Amano: Amano 2 [CHE-2] — Pilot

Cholesterol Esterase; Origin: Pseudomonas sp.
Amano: Amano 3 [CHE-3] — Pilot

Cholesterol Esterase; Origin: Pseudomonas sp.
Asahi — Pilot

Cholesterol Esterase; Origin: Pseudomonas sp.
Toyobo — Pilot

Tannase. **3.1.1.20**
Digallate + H(2)O = 2 gallate.

Tannase; Origin: Aspergillus ficuum
Jülich Enzyme Products — Lab

Lipoprotein lipase. **3.1.1.34**
Clearing factor lipase. Diglyceride lipase. Diacylglycerol lipase.
Triacylglycerol + H(2)O = diacylglycerol + a fatty acid anion.

Lipoprotein Lipase; Origin: Chromobacterium viscosum
Fluka — Pilot

Lipoprotein Lipase; Origin: Pseudomonas fluorescens
Fluka — Pilot

Lipoprotein Lipase; Origin: Pseudomonas sp.
Fluka — Pilot

Alkaline phosphatase. **3.1.3.1**
Alkaline phosphomonoesterase. Phosphomonoesterase. Glycerophosphatase.
An orthophosphoric monoester + H(2)O = an alcohol + phosphate.

Phosphatase, alkaline
Seravac — Pilot

Phosphatase, alkaline; Origin: Bacillus sp.
Biocatalysts — Pilot

Phosphatase alkaline; Origin: bovine intestinal mucosa
Fluka — Industrial

Phosphatase alkaline; Origin: calf intestinal mucosa
Fluka — Industrial

Phosphatase alkaline, immobilized on Agarose; Origin: calf intestinal mucosa
Fluka — Industrial

Phosphatase alkaline, immobilized on Agarose; Origin: calf intestinal mucosa
Fluka — Industrial

Phosphatase, alkaline; Origin: calf intestine
Roche Diagnostics: Phosphatase, alkaline, EIA Grade — Industrial

Phosphatase, alkaline; Origin: calf intestine or kidney
Biozyme — Pilot

Phosphatase, alkaline, highly active; Origin: calf intestine, rec. in Pichia pastoris
Roche Diagnostics: Phosphatase, alkaline, EIA Grade, highly active — Industrial

Phosphatase, alkaline; Origin: E. coli
Fluka — Lab

Phosphatase, alkaline ; Origin: Escherichia coli
Asahi — Pilot

Phosphatase, alkaline; Origin: microorganisms
Unitika — Pilot

Table 20.5. (cont.).

Acid phosphatase. Acid phosphomonoesterase. Phosphomonoesterase. Glycerophosphatase.	**3.1.3.2** An orthophosphoric monoester + H(2)O = an alcohol + phosphate.
Phosphatase, acid; Origin: potato Roche Diagnostics: Phosphatase, acid, grade II	Pilot
Phosphatase, acid; Origin: potatoes Fluka	Pilot
3-phytase. Phytase. Phytate 3-phosphatase. Myo-inositol-hexaphosphate 3-phosphohydrolase.	**3.1.3.8** Myo-inositol hexakisphosphate + H(2)O = D-myo-inositol1,2,4,5,6-pentakisphosphate + phosphate.
Phytase; Origin: Aspergillus niger Amano	Industrial
Phytase; Origin: Peniophora lycii, rec. In Asp. oryzae Novozymes: Bio-feed® Phytase	Industrial
Phospholipase C. Lipophosphodiesterase I. Lecithinase C. Clostridium welchii alpha-toxin. Clostridium oedematiens beta- and gamma-toxins.	**3.1.4.3** A phosphatidylcholine + H(2)O = 1,2-diacylglycerol + cholinephosphate.
Phospholipase C; Origin: Bacillus cereus Asahi	Pilot
Phospholipase C; Origin: Bacillus cereus Fluka	Pilot
Phospholipase C; Origin: Clostridium perfringens Fluka	Pilot
Phospholipase D. Lipophosphodiesterase II. Lecithinase D. Choline phosphatase.	**3.1.4.4** A phosphatidylcholine + H(2)O = choline + a phosphatidate.
Glycerophosphorylcholine Phosphodiesterase; Origin: microorganisms Asahi	Pilot
Phospholipase D; Origin: Streptomyces chromofuscus Asahi	Pilot
Phospholipase D; Origin: Streptomyces chromofuscus Fluka	Pilot
Phospholipase D; Origin: Streptomyces sp. Asahi	Pilot
Sphingomyelin phosphodiesterase. Acid sphingomyelinase. Neutral sphingomyelinase.	**3.1.4.12** Sphingomyelin + H(2)O = N-acylsphingosine + choline phosphate.
Sphingomyelinase; Origin: Streptomyces sp. Asahi	Pilot
Deoxyribonuclease I. Pancreatic DNase. DNase. Thymonuclease.	**3.1.21.1** Endonucleolytic cleavage to 5'-phosphodinucleotide and5'-phosphooligonucleotide end-products.
Deoxyribonuclease I; Origin: bovine pancreas Fluka	Pilot
Ribonuclease T1. Guanyloribonuclease. Aspergillus oryzae ribonuclease. RNase N1. RNase N2.	**3.1.27.3** Two-stage endonucleolytic cleavage to 3'-phosphomononucleotides and3'-phosphooligonucleotides ending in G-P with 2',3'-cyclic phosphateintermediates.
Ribonuclease T1; Origin: Aspergillus oryzae Fluka	Lab

Table 20.5. (cont.).

Pancreatic ribonuclease. RNase. RNase I. RNase A. Pancreatic RNase.	**3.1.27.5** Endonucleolytic cleavage to 3'-phosphomononucleotides and 3'-phosphooligonucleotides ending in C-P or U-P with 2',3'-cyclicphosphate intermediates.
Ribonuclease; Origin: beef pancreas Biozyme	Pilot
Ribonuclease A; Origin: bovine pancreas Fluka	Pilot
Ribonuclease, immobilized on EupergitC; Origin: bovine pancreas Fluka	Lab
Aspergillus nuclease S1. Endonuclease S1. Single-stranded-nucleate endonuclease. Deoxyribonuclease S1.	**3.1.30.1** Endonucleolytic cleavage to 5'-phosphomononucleotide and 5'-phosphooligonucleotide end-products.
Nuclease; Origin: Penicillium citrinum Amano: Enzyme RP-1	Industrial
Nuclease P1; Origin: Penicillium citrinum Fluka	Lab
Micrococcal nuclease. Micrococcal endonuclease.	**3.1.31.1** Endonucleolytic cleavage to 3'-phosphomononucleotide and 3'-phosphooligonucleotide end-products.
Nuclease micrococcal; Origin: Staphylococcus aureus Fluka	Lab
Alpha-amylase. 1,4-alpha-D-glucan glucanohydrolase. Glycogenase.	**3.2.1.1** Endohydrolysis of 1,4-alpha-glucosidic linkages in oligosaccharides and polysaccharides.
Amylase; Origin: Aspergillus niger Amano: Gluczyme NL4.2	Industrial
alpha-Amylase; Origin: Aspergillus oryzae Fluka	Industrial
Amylase; Origin: Aspergillus oryzae Amano: Amylase DS	Industrial
Amylase; Origin: Aspergillus oryzae Amano: Biozyme F10 SD	Industrial
Amylase; Origin: Aspergillus oryzae Amano: Biozyme S Conc.	Industrial
Taka-Diastase; Origin: Aspergillus oryzae Fluka	Pilot
alpha-Amylase; Origin: Bacillus amyloliquefaciens Fluka	Industrial
alpha-Amylase; Origin: Bacillus licheniformis Fluka	Industrial
alpha-Amylase; Origin: Bacillus subtilis Fluka	Industrial
Amylase; Origin: Bacillus subtilis Amano: Amylase A "Amano" Conc.	Industrial
alpha-Amylase; Origin: fungus Novozymes: Fungamyl®	Industrial
alpha-Amylase; Origin: hog pancreas Fluka	Lab

Table 20.5. (cont.).

Amylase; Origin: microbacterium Amano: AMT "Amano"	Industrial
Amylase; Origin: microorganisms Novozymes: Aquazym®	Industrial
Amylase; Origin: microorganisms Novozymes: BAN (Bacterial Amylase Novo)	Industrial
alpha-Amylase; Origin: rec. microorganism Novozymes: Duramyl™	Industrial
alpha-Amylase; Origin: rec. microorganism Novozymes: Liquozyme®	Industrial
alpha-Amylase; Origin: rec. microorganism Novozymes: Termamyl®	Industrial
alpha-Amylase; Origin: rec. microorganism Novozymes: Termamyl, Type LS	Industrial
alpha-Amylase; Origin: rec. microorganism Novozymes: Thermozyme™	Industrial
Amylase; Origin: Rhizopus niveus Amano: Gluczyme 12	Industrial
Beta-amylase. 1,4-alpha-D-glucan maltohydrolase. Saccharogen amylase. Glycogenase.	**3.2.1.2** Hydrolysis of 1,4-alpha-glucosidic linkages in polysaccharides so asto remove successive maltose units from the non-reducing ends of the chains.
beta-Amylase; Origin: barley Fluka	Pilot
beta-Amylase; Origin: sweet potato Fluka	Pilot
Glucan 1,4-alpha-glucosidase. Glucoamylase. 1,4-alpha-D-glucan glucohydrolase. Amyloglucosidase. Gamma-amylase.	**3.2.1.3** Hydrolysis of terminal 1,4-linked alpha-D-glucose residues successively from non-reducing ends of the chains with release ofbeta-D-glucose.
Amyloglucosidase; Origin: Aspergillus niger Fluka	Industrial
Amyloglucosidase; Origin: rec. microorganism Novozymes: AMG	Industrial
Cellulase. Endoglucanase. Endo-1,4-beta-glucanase. Carboxymethyl cellulase.	**3.2.1.4** Endohydrolysis of 1,4-beta-D-glucosidic linkages in cellulose.
Cellulase Novozymes: Novozym® 342	Industrial
Cellulase; Origin: Aspergillus niger Amano: Cellulase A "Amano" 3	Industrial
Cellulase; Origin: Aspergillus niger Amano: Cellulase DS	Industrial
Cellulase; Origin: Aspergillus niger Fluka	Industrial
Cellulase; Origin: fungus Novozymes: Celluzyme®	Industrial
Cellulase; Origin: Humicola insolens Fluka	Lab

Table 20.5. (cont.).

Cellulase; Origin: rec. microorganism Novozymes: Carezyme®	Industrial
Cellulase; Origin: rec. microorganism Novozymes: DeniMax®	Industrial
Cellulase; Origin: Trichoderma longibrachiatum Fluka	Industrial
Cellulase; Origin: Trichoderma reesei Fluka	Lab
Cellulase; Origin: Trichoderma viride Amano: Cellulase T "Amano" 4	Industrial
Cellulase; Origin: Trichoderma viride Jülich Enzyme Products	Lab
Endo-1,3(4)-beta-glucanase. Endo-1,4-beta-glucanase. Endo-1,3-beta-glucanase. Laminarinase.	**3.2.1.6** Endohydrolysis of 1,3- or 1,4-linkages in beta-D-glucans when the glucose residue whose reducing group is involved in the linkage to be hydrolysed is itself substituted at C-3.
beta-glucanase Novozymes: Cereflo®	Industrial
beta-glucanase Novozymes: Finizym®	Industrial
beta-glucanase, heat-stable Novozymes: Ultraflo®	Industrial
beta-Glucanase; Origin: Aspergillus niger Fluka	Industrial
beta-Glucanase; Origin: Bacillus subtilis Fluka	Industrial
Inulinase. Inulase.	**3.2.1.7** Endohydrolysis of 2,1-beta-D-fructosidic linkages in inulin.
Inulinase; Origin: Aspergillus niger Fluka	Lab
Endo-1,4-beta-xylanase. 1,4-beta-D-xylan xylanohydrolase.	**3.2.1.8** Endohydrolysis of 1,4-beta-D-xylosidic linkages in xylans.
Xylanase Novozymes: Pulpzyme™ HC	Industrial
Xylanase; Origin: Aspergillus niger Amano: Hemicellulase "Amano" 90	Industrial
Xylanase; Origin: Aspergillus niger Amano: Hemicellulase "Amano" 90	Industrial
Xylanase; Origin: bacteria Fluka	Industrial
Xylanase; Origin: rec. microorganism Novozymes: Pentopan™ Mono	Industrial
Xylanase; Origin: rec. microorganism Novozymes: Shearzyme™	Industrial
Xylanase; Origin: Trichoderma viride Fluka	Lab

Table 20.5. (cont.).

Dextranase.	**3.2.1.11**
Alpha-1,6-glucan-6-glucanohydrolase.	Endohydrolysis of 1,6-alpha-D-glucosidic linkages in dextran.
Dextranase	
Novozymes: Dextranase	Industrial
Dextranase; Origin: Chaetomium erraticum)	
Amano: Dextranase L "Amano"	Industrial
Dextranase; Origin: Paecilomyces lilacinus	
Fluka	Pilot
Chitinase.	**3.2.1.14**
Chitodextrinase. 1,4-beta-poly-N-acetylglucosaminidase. Poly-beta-glucosaminidase.	Hydrolysis of the 1,4-beta-linkages of N-acetyl-D-glucosamine polymers of chitin.
Chitinase; Origin: bean leaves	
Jülich Enzyme Products: Chitinase BB	Lab
Chitinase; Origin: Streptomyces griseus	
Fluka	Pilot
Chitinase; Origin: sugar beet	
Fluka	Lab
Chitinase; Origin: sugar-beet leaves	
Jülich Enzyme Products: Chitinase ZR	Lab
Polygalacturonase.	**3.2.1.15**
Pectin depolymerase. Pectinase.	Random hydrolysis of 1,4-alpha-D-galactosiduronic linkages in pectate and other galacturonans.
Pectinase; Origin: Aspergillus niger	
Fluka	Industrial
Pectinase; Origin: mould	
Fluka	Industrial
Pectinase; Origin: Rhizopus sp.	
Fluka	Industrial
Lysozyme.	**3.2.1.17**
Muramidase.	Hydrolysis of the 1,4-beta-linkages between N-acetyl-D-glucosamine and N-acetylmuramic acid in peptidoglycan heteropolymers of the prokaryotes cell walls.
Lysozyme	
Seravac	Pilot
Lysozyme; Origin: chicken egg white	
Biozyme	Industrial
Lysozyme; Origin: hen egg white	
Fluka	Industrial
Exo-alpha-sialidase.	**3.2.1.18**
Sialidase. Neuraminidase. N-acylneuraminate glycohydrolase. Alpha-neuraminidase.	Hydrolysis of alpha-(2->3)-, alpha-(2->6)-, alpha-(2->8)-glycosidic linkages of terminal sialic residues in oligosaccharides, glycoproteins, glycolipids, colominic acid and synthetic substrates.
Neuraminidase; Origin: Clostridium perfringens	
Fluka	Pilot
Neuraminidase, immobilized on Agarose4B; Origin: Clostridium perfringens	
Fluka	Pilot
Neuraminidase; Origin: microorganisms	
Unitika	Pilot

Table 20.5. (cont.).

Neuraminidase; Origin: Streptococcus sp.
Toyobo — Pilot

Neuraminidase; Origin: Vibrio cholerae
Fluka — Lab

Alpha-glucosidase. — **3.2.1.20**
Maltase. Glucoinvertase. Glucosidosucrase. Maltase-glucoamylase. — Hydrolysis of terminal, non-reducing 1,4-linked D-glucose residues with release of D-glucose.

alpha-Glucosidase; Origin: Aspergillus niger
Fluka — Industrial

alpha-Glucosidase; Origin: Bacillus stearothermophilus
Unitika: alpha-Glucosidase (alpha-Glu) — Pilot

alpha-Glucosidase; Origin: microorganisms
Toyobo — Pilot

alpha-Glucosidase; Origin: yeast
Biozyme — Pilot

alpha-Glucosidase; Origin: yeast
Fluka — Lab

alpha-Glucosidase; Origin: yeast overproducer
Roche Diagnostics: alpha-Glucosidase (alpha-Gluc) — Industrial

Beta-glucosidase. — **3.2.1.21**
Gentobiase. Cellobiase. Amygdalase. — Hydrolysis of terminal, non-reducing beta-D-glucose residues with release of beta-D-glucose.

Beta-Glucosidase
Seravac — Pilot

beta-Glucosidase; Origin: almonds
Fluka — Pilot

beta-Glucosidase; Origin: sweet almond
Toyobo — Pilot

beta-Glucosidase; Origin: sweet almonds
Biozyme — Pilot

Alpha-galactosidase. — **3.2.1.22**
Melibiase. — Melibiose + H(2)O = galactose + glucose.

alpha-Galactosidase; Origin: rec. microorganism
Novozymes: Alpha-gal™ — Industrial

Beta-galactosidase. — **3.2.1.23**
Lactase. — Hydrolysis of terminal, non-reducing beta-D-galactose residues in beta-D-galactosides.

Lactase; Origin: Aspergillus oryzae
Amano: Lactase 14-DS — Industrial

Lactase; Origin: Aspergillus oryzae
Amano: Lactase F "Amano" — Industrial

beta-Galactosidase; Origin: E. coli
Fluka — Pilot

beta-Galactosidase; Origin: E. coli overproducer
Roche Diagnostics: beta-Galactosidase — Industrial

beta-Galactosidase; Origin: Escherichia coli
Toyobo — Pilot

beta-Galactosidase; Origin: Kluviromyces lactis
Recordati: beta-Galactosidase, Lattasi beads — Industrial

Table 20.5. (cont.).

beta-Galactosidase; Origin: Kluyveromyces fragilis
Fluka — Pilot

Lactase; Origin: Kluyveromyces lactis
Novozymes: Lactozym® — Industrial

beta-Galactosidase; Origin: microorganisms
Unitika: beta-Galactosidase (beta-Gal) — Pilot

Alpha-mannosidase. **3.2.1.24**

Hydrolysis of terminal, non-reducing alpha-D-mannose residues in alpha-D-mannosides.

alpha-Mannosidase
Seravac — Pilot

Beta-fructofuranosidase. **3.2.1.26**

Invertase. Saccharase.

Hydrolysis of terminal non-reducing beta-D-fructofuranoside residues in beta-D-fructofuranosides.

Invertase; Origin: Saccharomyces cerevisiae
Fluka — Industrial

Beta-glucuronidase. **3.2.1.31**

A beta-D-glucuronoside + H(2)O = an alcohol + D-glucuronate.

Beta-Glucuronidase
Seravac — Pilot

beta-Glucuronidase; Origin: bovine liver
Fluka — Lab

beta-Glucuronidase; Origin: E. coli
Fluka — Lab

beta-Glucuronidase; Origin: E. coli K12
Fluka — Lab

beta-Glucuronidase; Origin: Helix pomatia
Fluka — Lab

Hyaluronoglucosaminidase. **3.2.1.35**

Hyaluronidase.

Random hydrolysis of 1,4-linkages between N-acetyl-beta-D-glucosamine and D-glucuronate residues in hyaluronate.

Hyaluronidase
Seravac — Pilot

Hyaluronidase; Origin: bovine testes
Fluka — Lab

Hyaluronidase; Origin: ovine or bovine testes
Biozyme — Pilot

Hyaluronidase; Origin: sheep testes
Fluka — Lab

Hyaluronidase; Origin: Streptomyces hyalurolyticus
Fluka — Lab

Hyaluronidase; Origin: Streptomyces sp.
Amano: Amano 1 [HY-1] — Pilot

Hyaluronidase; Origin: Streptomyces sp.
Amano: Amano 3 [HY-3] — Pilot

Table 20.5. (cont.).

Glucan endo-1,3-beta-D-glucosidase. (1->3)-beta-glucan endohydrolase. Endo-1,3-beta-glucanase. Laminarinase.	**3.2.1.39** Hydrolysis of 1,3-beta-D-glucosidic linkages in 1,3-beta-D-glucans.
beta-1,3-D-Glucanase; Origin: Helix pomatia Fluka	Pilot
Alpha-dextrin endo-1,6-alpha-glucosidase. Pullulanase. Pullulan 6-glucanohydrolase. Limit dextrinase. Debranching enzyme.	**3.2.1.41** Starch-debranching enzyme, hydrolyses (1-6)-alpha-glucosidic linkages in pullulan and starch to form maltotriose.
Pullulanase; Origin: Bacillus sp. Amano: Debranchingenzyme "Amano" 8	Industrial
Pullulanase; Origin: Bacillus sp. Fluka	Industrial
Pullulanase; Origin: rec. microorganism Novozymes: Promozyme®	Industrial
Beta-N-acetylhexosaminidase. Beta-hexosaminidase. Hexosaminidase. N-acetyl-beta-glucosaminidase.	**3.2.1.52** Hydrolysis of terminal non-reducing N-acetyl-D-hexosamine residues in N-acetyl-beta-D-hexosaminides.
beta-N-Acetylglucosaminidase; Origin: jack bean Fluka	Lab
Agarase.	**3.2.1.81** Hydrolysis of 1,3-beta-D-galactosidic linkages in agarose, giving the tetramer as the predominant product.
Agarase; Origin: Pseudomonas atlantica Fluka	Lab
Thioglucosidase. Myrosinase. Sinigrinase. Sinigrase.	**3.2.3.1** A thioglucoside + H(2)O = a thiol + sugar.
Myrosinase; Origin: Senapis alba (white mustard seed) Biocatalysts	Pilot
Epoxide hydrolase. Epoxide hydratase. Arene-oxide hydratase.	**3.3.2.3** An epoxide + H(2)O = a glycol.
Epoxide Hydrolase; Origin: Agrobacterium sp. Fluka	Lab
Epoxide Hydrolase; Origin: Aspergillus niger Fluka	Lab
Epoxide Hydrolase; Origin: Rhodococcus rhodochrous Fluka	Lab
Epoxide Hydrolase; Origin: Rhodotorula glutinis Fluka	Lab
Hydrolases. **Acting on peptide bonds (peptide hydrolases).**	**3.4.-.-**
Protease; Origin: Aspergillus melleus Amano: Protease DS	Industrial
Protease; Origin: Aspergillus melleus Amano: Protease P "Amano" 6	Industrial
Protease; Origin: Aspergillus niger Altus	Industrial
Protease; Origin: Aspergillus niger Amano: Acid Protease A	Industrial

Table 20.5. (cont.).

Protease; Origin: Aspergillus niger Amano: Acid Protease DS	Industrial
Protease; Origin: Aspergillus niger Jülich Enzyme Products	Lab
Protease ; Origin: Aspergillus oryzae Altus	Industrial
Protease; Origin: Aspergillus oryzae Altus	Industrial
Protease; Origin: Aspergillus oryzae Altus	Industrial
Protease; Origin: Aspergillus oryzae Amano: Protease A "Amano" 2G	Industrial
Protease; Origin: Aspergillus oryzae Amano: Protease A-DS	Industrial
Protease; Origin: Aspergillus oryzae Amano: Protease M "Amano"	Industrial
Protease; Origin: Aspergillus oryzae Jülich Enzyme Products	Lab
Proteinase 2A; Origin: Aspergillus oryzae Fluka	Industrial
Protease; Origin: Aspergillus sp. Altus	Industrial
Protease, neutral; Origin: Bacillus amyloliquefaciens Novozymes: Neutrase®	Industrial
Protease; Origin: Bacillus licheniformis Novozymes: Bio-Feed® Pro	Industrial
Protease; Origin: Bacillus licheniformis Novozymes: Novozym® FM	Industrial
Protease, Endopeptidase ; Origin: Bacillus licheniformis Novozymes: Alcalase®	Industrial
Proteinase; Origin: Bacillus licheniformis Fluka	Industrial
Protease; Origin: Bacillus sp. Altus	Industrial
Protease; Origin: Bacillus sp. Novozymes: NovoCor S	Industrial
Protease, alkaline; Origin: Bacillus sp. Novozymes: Esperase®	Industrial
Proteinase, neutral; Origin: Bacillus sp. Toyobo	Industrial
Endoproteinase; Origin: Bacillus sp., rec. Fluka	Pilot
Protease; Origin: Bacillus sp., rec. Novozymes: Novo-Pro™ D	Industrial
Protease; Origin: Bacillus sp., rec. Novozymes: Pyrase®	Industrial
Protease; Origin: Bacillus stearothermophilus Amano: Protease S "Amano"	Industrial
Protease; Origin: Bacillus subtilis Amano: Proleather FG-F	Industrial

Table 20.5. (cont.).

Protease; Origin: Bacillus subtilis Amano: Protease N "Amano"	Industrial
Protease; Origin: Bacillus subtilis Amano: Protease NL "Amano"	Industrial
Protease; Origin: Bacillus subtilis Jülich Enzyme Products	Lab
Proteinase; Origin: Bacillus subtilis Fluka	Industrial
Proteinase; Origin: Bacillus subtilis var. biotecus A Fluka	Industrial
Protease; Origin: Carica papaya L. Amano: Papain W-40	Industrial
Protease, Proline-Specific Endopeptidase; Origin: Flavobacterium sp. Toyobo	Pilot
Protease, neutral to acidic; Origin: Fungus Novozymes: NovoCor P	Industrial
Protease, alkaline; Origin: microorganism, rec. in Bacillus sp. Novozymes: Savinase®	Industrial
Protease; Origin: microorganisms DSM Gist-brocades: Fermizyme	Industrial
Protease; Origin: Penicillum sp. Altus	Industrial
Protease; Origin: Protein engineered in rec. Bacillus Novozymes: Kannase™	Industrial
Protease, alkaline; Origin: Protein engineered, rec. in Bacillus sp. Novozymes: Everlase®	Industrial
Protease; Origin: Rhizomucor miehei, rec. in Aspergillus oryzae DSM Gist-brocades: Optiren	Industrial
Protease, neutral to acidic; Origin: Rhizomucor sp. Novozymes: NovoCor® AB	Industrial
Protease; Origin: Rhizopus niveus Amano: Acid Protease	Industrial
Protease; Origin: Rhizopus oryzae Amano: Peptidase R	Industrial
Pronase; Origin: Streptomyces griseus Fluka	Industrial
Pronase; Origin: Streptomyces griseus Fluka	Industrial
Pronase nonspecific protease; Origin: Streptomyces griseus Roche Diagnostics: Pronase nonspecific protease	Industrial
Protease, alkalophilic; Origin: Streptomyces sp. Toyobo	Pilot

Leucyl aminopeptidase. 3.4.11.1

Cytosol aminopeptidase. Leucine aminopeptidase. Peptidase S.

Release of an N-terminal amino acid, Xaa-l-Xbb-, in which Xaa is preferably Leu, but may be other amino acids including Pro although not Arg or Lys, and Xbb may be Pro.

Leucine Aminopeptidase, cytosol; Origin: hog kidney Fluka	Lab
Leucine aminopeptidase; Origin: pig kidney Biozyme	Pilot

Table 20.5. (cont.).

Xaa-Pro dipeptidase.	**3.4.13.9**
X-Pro dipeptidase. Proline dipeptidase. Imidodipeptidase. Prolidase.	Hydrolysis of Xaa-l-Pro dipeptides; also acts on aminoacyl-hydroxyproline analogs. No action on Pro-l-Pro.
Prolidase; Origin: Lactococcus lactis	
Fluka	Lab
Prolidase; Origin: pig kidney	
Biozyme	Pilot
Dipeptidyl-peptidase I.	**3.4.14.1**
Cathepsin C. Cathepsin J. Dipeptidyl aminopeptidase I. Dipeptidyl transferase.	Release of an N-terminal dipeptide, Xaa-Xbb-l-Xcc, except when Xaa is Arg or Lys, or Xbb or Xcc is Pro.
Cathepsin C, sol.; Origin: bovine spleen	
Roche Diagnostics: Cathepsin C, sol.	Industrial
Transferred entry: 3.4.16.5 and 3.4.16.6.	**3.4.16.1**
Carboxypeptidase Y; Origin: baker's yeast	
Fluka	Lab
Carboxypeptidase Y; Origin: yeast	
Roche Diagnostics: Carboxypeptidase Y, Sequencing Grade	Lab
Carboxypeptidase A.	**3.4.17.1**
Carboxypolypeptidase.	Peptidyl-L-amino acid + H(2)O = peptide + L-amino acid.
Carboxypeptidase A	
Seravac	Pilot
Carboxypeptidase A; Origin: bovine pancreas	
Fluka	Lab
Membrane Pro-X carboxypeptidase.	**3.4.17.16**
Carboxypeptidase P. Microsomal carboxypeptidase.	Release of a C-terminal residue other than proline, by preferentialcleavage of prolyl bond.
Carboxypeptidase P; Origin: Penicillium janthinellum	
Fluka	Lab
Pyroglutamyl-peptidase I.	**3.4.19.3**
5-oxoprolyl-peptidase. Pyrrolidone-carboxylate peptidase. Pyrrolidone carboxyl peptidase. Pyroglutamyl aminopeptidase.	5-oxoprolyl-peptide + H(2)O = 5-oxoproline + peptide.
Pyroglutamate Aminopeptidase; Origin: calf liver	
Fluka	Pilot
Hydrolases.	**3.4.21.-**
Acting on peptide bonds (peptide hydrolases).	
Serine endopeptidases.	
Endoproteinase Pro-C; Origin: microorganism, rec. in E. coli	
Fluka	Lab
Chymotrypsin.	**3.4.21.1**
Chymotrypsin A. Chymotrypsin B. Alpha-chymotrypsin.	Preferential cleavage: Tyr-l-Xaa, Trp-l-Xaa, Phe-l-Xaa, Leu-l-Xaa.
alpha-Chymotrypsin	
Seravac	Pilot
alpha-Chymotrypsin; Origin: Bacillus licheniformis	
Altus	Industrial
alpha-Chymotrypsin; Origin: bovine pancreas	
Fluka	Pilot

Table 20.5. (cont.).

Trypsin. Alpha- and beta-trypsin.	**3.4.21.4** Preferential cleavage: Arg-l-Xaa, Lys-l-Xaa.
Trypsin Seravac	Pilot
Trypsin Seravac	Pilot
Trypsin; Origin: bovine pancreas Fluka	Industrial
Trypsin; Origin: pig Pancreas Biozyme	Industrial
Trypsin ; Origin: porcine pancreas Altus	Industrial
Trypsin; Origin: porcine pancreas Biocatalysts: Trypsin	Industrial
Trypsin; Origin: porcine pancreas Biocatalysts: Trypsin 250	Industrial
Trypsin; Origin: porcine pancreas Biocatalysts: Trypsin 250	Industrial
Trypsin; Origin: porcine pancreas Novozymes: Crystalline Porcine Trypsin	Industrial
Trypsin (Chrymotrypsin as minor constituent); Origin: porcine pancreas Novozymes: PTN (Pancreatic Trypsin Novo)	Industrial
Thrombin. Fibrinogenase.	**3.4.21.5** Preferential cleavage: Arg-l-Gly; activates fibrinogen to fibrin andreleases fibrinopeptide A and B.
Thrombin; Origin: bovine plasma Fluka	Pilot
Enteropeptidase. Enterokinase.	**3.4.21.9** Selective cleavage of 6-Lys-l-Ile-7 bond in trypsinogen.
Enteropeptidase Seravac	Pilot
Glutamyl endopeptidase. Staphylococcal serine proteinase. V8 proteinase. Protease V8. Endoproteinase Glu-C.	**3.4.21.19** Preferential cleavage: Asp-l-Xaa, Glu-l-Xaa.
Endoprotease Glu-C; Origin: Endophrins Altus	Pilot
Endoproteinase Glu-C; Origin: Staphylococcus aureus strain V8 Fluka	Lab
Endoproteinase Glu-C; Origin: Staphylococcus aureus strain V8 Roche Diagnostics: Endoproteinase Glu-C, Sequencing Grade	Lab
Pancreatic elastase. Pancreatopeptidase E. Pancreatic elastase I.	**3.4.21.36** Hydrolysis of proteins, including elastin. Preferential cleavage:Ala-l-Xaa.
Elastase; Origin: hog pancreas Fluka	Pilot
Elastase; Origin: pig pancreas Biozyme	

Table 20.5. (cont.).

Elastase; Origin: porcine pancreas
Altus — Industrial

Lysyl endopeptidase. — **3.4.21.50**
Achromobacter proteinase I. Lysyl bond specific proteinase.
Preferential cleavage: Lys-|-Xaa, including Lys-|-Pro.

Endoproteinase Lys-C; Origin: Lysobacter enzymogenes
Fluka — Lab

Endoproteinase Lys-C; Origin: Lysobacter enzymogenes
Roche Diagnostics: Endoproteinase Lys-C, Sequencing Grade — Lab

Subtilisin. — **3.4.21.62**
Hydrolysis of proteins with broad specificity for peptide bonds, and a preference for a large uncharged residue in P1. Hydrolyses peptide amides.

Protease, Subtilisin Carlsberg; Origin: Bacillus licheniformis
Novozymes: Subtilisin A — Pilot

Subtilisin; Origin: Bacillus licheniformis
Altus: ChiroCLEC-BL (dry) — Industrial

Subtilisin; Origin: Bacillus licheniformis
Altus: ChiroCLEC-BL (slurry) — Industrial

Subtilisin; Origin: Bacillus licheniformis
Altus: PeptiCLEC-BL (dry) — Industrial

Subtilisin; Origin: Bacillus licheniformis
Altus: PeptiCLEC-BL (slurry) — Industrial

Subtilisin; Origin: Bacillus licheniformis
Fluka — Industrial

Subtilisin; Origin: Bacillus licheniformis
Roche Diagnostics: CHIRAZYME P-1, lyo. — Industrial

Subtilisin; Origin: Bacillus licheniformis
Roche Diagnostics: CHIRAZYME P-1, sol. — Industrial

Subtilisin Carlsberg; Origin: Bacillus licheniformis
Altus — Industrial

Proteinase K. — **3.4.21.64**
Endopeptidase K. Tritirachium alkaline proteinase. Tritirachium album proteinase K.
Hydrolysis of keratin and of other proteins, with subtilisin-like specificity. Hydrolyses peptides amides.

Proteinase N; Origin: Bacillus subtilis
Fluka — Industrial

Proteinase K; Origin: Tritirachium album
Fluka — Industrial

Proteinase K, immobilized on EupergitC; Origin: Tritirachium album
Fluka — Lab

Proteinase K, lyo.; Origin: Tritirachium album
Roche Diagnostics: Proteinase K, lyo. — Industrial

Papain. — **3.4.22.2**
Papaya peptidase I.
Hydrolysis of proteins with broad specificity for peptide bonds, with preference for a residue bearing a large hydrophobic sidechain at the P2 position. Does not accept Val at P1'.

Papain
Biocatalysts: PROMOD 144L — Industrial

Papain; Origin: Carica papaya
Fluka — Industrial

Table 20.5. (cont.).

Papain; Origin: Carica papaya
Roche Diagnostics: Papain — Industrial

Papain, immobilized on Eupergit® C; Origin: Carica papaya
Fluka — Lab

Papain ; Origin: Papaya Latex
Altus — Industrial

Chymopapain. — **3.4.22.6**
Papaya proteinase II. — Specificity similar to that of papain.

Chymopapain; Origin: Papaya Latex
Altus — Industrial

Clostripain. — **3.4.22.8**
Clostridiopeptidase B. — Preferential cleavage: Arg-l-Xaa, including Arg-l-Pro bond, but not Lys-l-Xaa.

Clostripain; Origin: Clostridium histolyticum
Altus — Industrial

Clostripain; Origin: Clostridium histolyticum
Fluka — Pilot

Endoproteinase Arg-C; Origin: Clostridium histolyticum
Roche Diagnostics: Endoproteinase Arg-C, Sequencing Grade — Lab

Clostripain rec.; Origin: E. coli
Fluka — Pilot

Endoproteinase Arg-C; Origin: mice (submaxillary glands)
Fluka — Lab

Stem bromelain. — **3.4.22.32**
Bromelain. — Broad specificity for cleavage of proteins, but strong preference for Z-Arg-Arg-l-NHMec amongst small molecule substrates.

Bromelain; Origin: pinapple stem
Altus — Industrial

Bromelain; Origin: pineapple stem
Fluka — Pilot

Pepsin A. — **3.4.23.1**
Pepsin. — Preferential cleavage: hydrophobic, preferably aromatic, residues in PI and P1' positions. Cleaves 1-Phe-l-Val-2, 4-Gln-l-His-5, 13-Glu-l-Ala-14, 14-Ala-l-Leu-15, 15-Leu-l-Tyr-16,

Pepsin; Origin: hog stomach
Fluka — Industrial

Pepsin; Origin: pig stomach mucosa
Biozyme — Pilot

Pepsin ; Origin: porcine pancreas
Altus — Industrial

Chymosin. — **3.4.23.4**
Rennin. — Broad specificity similar to that of pepsin A. Clots milk by cleavageof a single bond in casein (kappa chain).

Chymosin; Origin: calf stomach
Altus — Industrial

Table 20.5. (cont.).

Microbial collagenase. Clostridium histolyticum collagenase. Clostridiopeptidase A. Collagenase A. Collagenase I.	**3.4.24.3** Digestion of native collagen in the triple helical region at Xaa-l-Gly bonds. With synthetic peptides, a preference is shown for Gly at P3 and P1'; Pro and Ala at P2 and P2'; and hydroxyproline, Ala or Arg
Collagenase; Origin: Clostridium histolyticum Fluka	Pilot
Collagenase; Origin: Clostridium histolyticum Fluka	Pilot
Collagenase; Origin: Clostridium sp. Amano: Amano 1 [CL-1]	Pilot
Collagenase; Origin: Clostridium sp. Amano: Amano S [CL-S]	Pilot
Thermolysin. Bacillus thermoproteolyticus neutral proteinase.	**3.4.24.27** Preferential cleavage: Xaa-l-Leu > Xaa-l-Phe.
Thermolysin; Origin: Bacillus thermoproteolyticus Altus: PeptiCLEC-TR (dry)	Industrial
Thermolysin; Origin: Bacillus thermoproteolyticus Altus: PeptiCLEC-TR (slurry)	Industrial
Thermolysin; Origin: Bacillus thermoproteolyticus Fluka	Pilot
Peptidyl-Asp metalloendopeptidase. Endoproteinase Asp-N.	**3.4.24.33** Cleavage of Xaa-l-Asp, Xaa-l-Glu and Xaa-l-cysteic acid bonds.
Endoproteinase Asp-N; Origin: Pseudomonas fragi (mutant) Fluka	Lab
Endoproteinase Asp-N; Origin: Pseudomonas fragi (mutant) Roche Diagnostics: Endoproteinase Asp-N, Sequencing Grade	Lab
Hydrolases. **Acting on carbon-nitrogen bonds, other than peptide bonds.**	**3.5.-.-**
Peptide Amidase; Origin: Citrus sinensis Fluka	Lab
Hydrolases. **Acting on carbon-nitrogen bonds, other than peptide bonds.** **In linear amides.**	**3.5.1.-**
Glutaryl Acylase, immobilized Fluka	Industrial
Glutaryl acylase, carrier-fixed; Origin: E. coli overproducer Roche Diagnostics: Glutaryl acylase, carrier-fixed (Gl-Ac)	Industrial
Glutaryl-7-ACA Acylase; Origin: microorganism, rec. in E. coli Recordati: GAA Beads	Industrial
Asparaginase. L-asparaginase. L-asparagine amidohydrolase.	**3.5.1.1** L-asparagine + H(2)O = L-aspartate + NH(3).
L-Asparaginase; Origin: E. coli Fluka	Lab

Table 20.5. (cont.).

Glutaminase. L-glutamine amidohydrolase.	**3.5.1.2** L-glutamine + H(2)O = L-glutamate + NH(3).
Glutaminase; Origin: Bacillus subtilis Amano: Glutaminase F "Amano" 100	Industrial
Amidase. Acylamidase. Acylase.	**3.5.1.4** A monocarboxylic acid amide + H(2)O = a monocarboxylate + NH(3).
Amidase; Origin: Pseudomonas aeruginosa, rec. in E. coli Fluka	Lab
Urease.	**3.5.1.5** Urea + H(2)O = CO(2) + 2 NH(3).
Urease; Origin: jack bean Fluka	Industrial
Urease; Origin: jack bean Biozyme	Pilot
Urease; Origin: jack bean Roche Diagnostics: Urease	Industrial
Urease ; Origin: jack bean Seravac	Industrial
Urease; Origin: jack bean Toyobo	Pilot
Urease, immobilized on Eupergit C; Origin: jack bean Fluka	Lab
Penicillin amidase. Penicillin acylase.	**3.5.1.11** Penicillin + H(2)O = a fatty acid anion + 6-aminopenicillanate.
Penicillin Acylase; Origin: E. coli Altus	Industrial
Penicillin Acylase; Origin: E. coli Altus: ChiroCLEC-EC (dry)	Industrial
Penicillin Acylase; Origin: E. coli Altus: ChiroCLEC-EC (slurry)	Industrial
Penicillin Amidase; Origin: E. coli Fluka	Industrial
Penicillin Amidase, immobilized on Eupergit® C; Origin: E. coli Fluka	Industrial
Penicillin G Amidase, immobilized; Origin: E. coli Fluka	Industrial
Penicillin-G Acylase; Origin: E. coli Recordati: PGA beads, Standard	Industrial
Penicillin-G Acylase; Origin: E. coli Recordati: PGA beads, Superenzyme	Industrial
Penicillin G Amidase; Origin: E. coli overproducer Roche Diagnostics: Penicillin G Amidase (PGA-450)	Industrial
Aminoacylase. Histozyme. Hippuricase. Benzamidase. Dehydropeptidase II.	**3.5.1.14** An N-acyl-L-amino acid + H(2)O = a fatty acid anion + an L-amino acid.
Acylase I; Origin: Aspergillus melleus Fluka	Industrial

Table 20.5. (cont.).

Acylase I, immobilized on Eupergit C; Origin: Aspergillus sp.
Fluka — Pilot

Aminoacylase; Origin: Aspergillus sp.
Amano: Acylase — Industrial

Acylase I; Origin: hog kidney
Fluka — Pilot

Acylase I; Origin: pig kidney
Biozyme — Pilot

Acylase; Origin: Streptomyces chartreusis
Fluka — Lab

Acylase; Origin: Streptomyces griseocarneus
Fluka — Lab

Acylase; Origin: Streptomyces hachijoensis
Fluka — Lab

Acylase; Origin: Streptomyces toyocaensis
Fluka — Lab

Acylase; Origin: Streptomyces zaomyceticus
Fluka — Lab

Dihydropyrimidinase. **3.5.2.2**
Hydantoinase. 5,6-dihydrouracil + H(2)O = 3-ureidopropionate.

Hydantoinase; Origin: Agrobacterium radiobacter
Recordati: Hyda-REC — Industrial

D-Hydantoinase; Origin: Azuki beans
Fluka — Lab

D-Hydantoinase 1, carrier-fixed; Origin: Bacillus thermoglucosidasius, rec. in E. coli
Roche Diagnostics: D-Hydantoinase 1, carrier-fixed — Industrial

D-Hydantoinase, recombinant, immobilized; Origin: E. coli
Fluka — Industrial

Beta-lactamase. **3.5.2.6**
Penicillinase. Cephalosporinase. A beta-lactam + H(2)O = a substituted beta-amino acid.

beta-Lactamase I; Origin: Bacillus cereus
Fluka — Lab

beta-Lactamase; Origin: Enterobacter cloacae
Fluka — Lab

Creatininase. **3.5.2.10**
Creatinine amidohydrolase. Creatinine + H(2)O = creatine.

Creatininase; Origin: microorganisms
Asahi — Pilot

Arginase. **3.5.3.1**
Arginine amidinase. Canavanase. L-arginine + H(2)O = L-ornithine + urea.

Arginase
Biozyme: Bovine liver — Pilot

L-Arginase; Origin: bovine liver
Fluka — Lab

Table 20.5. (cont.).

Creatinase.	**3.5.3.3**
Creatine amidinohydrolase.	Creatine + H(2)O = sarcosine + urea.
Creatinase; Origin: Bacillus sp.	
Asahi	Pilot
Creatinase; Origin: Flavobacterium sp.	
Fluka	Pilot
Hydrolases.	**3.5.4.-**
Acting on carbon-nitrogen bonds, other than peptide bonds.	
In cyclic amidines.	
Deaminase; Origin: Aspergillus melleus	
Amano: Deamizyme 50000	Industrial
Adenosine deaminase.	**3.5.4.4**
Adenosine aminohydrolase.	Adenosine + H(2)O = inosine + NH(3).
Adenosine Deaminase; Origin: calf intestinal mucosa	
Fluka	Pilot
Adenosine Deaminase; Origin: calf intestine	
Roche Diagnostics: Adenosine Deaminase	Industrial
Nitrilase.	**3.5.5.1**
	A nitrile + H(2)O = a carboxylate + NH(3).
Nitrilase, broad-range; Origin: microorganism, rec. in E. coli	
BioCatalytics: NIT-101	Lab
Nitrilase, broad-range; Origin: microorganism, rec. in E. coli	
BioCatalytics: NIT-102	Lab
Nitrilase, broad-range; Origin: microorganism, rec. in E. coli	
BioCatalytics: NIT-103	Lab
Inorganic pyrophosphatase.	**3.6.1.1**
Inorganic diphosphatase. Pyrophosphate phosphohydrolase. Diphosphate phosphohydrolase.	Diphosphate + H(2)O = 2 phosphate.
Pyrophosphatase, inorganic; Origin: baker's yeast	
Fluka	Lab
Pyrophosphatase, inorganic; Origin: E. coli	
Fluka	Lab
Pyruvate decarboxylase.	**4.1.1.1**
Alpha-carboxylase. Pyruvic decarboxylase. Alpha-ketoacid carboxylase.	A 2-oxo acid = an aldehyde + CO(2).
Pyruvate Decarboxylase; Origin: Zymomonas mobils, E. coli (rec.)	
Jülich Enzyme Products	Lab
Oxaloacetate decarboxylase.	**4.1.1.3**
Oxalate beta-decarboxylase.	Oxaloacetate = pyruvate + CO(2).
Oxalacetate Decarboxylase; Origin: Pseudomonas sp.	
Fluka	Lab
Oxaloacetate Decarboxylase; Origin: Pseudomonas sp.	
Asahi	Pilot

Table 20.5. (cont.).

Acetolactate decarboxylase. **4.1.1.5**

(S)-2-hydroxy-2-methyl-3-oxobutanoate = (R)-2-acetoin + CO(2).

Acetolactate Decarboxylase; Origin: Bac.Brevis in rec, Bac. subtilis
Novozymes: Maturex® — Industrial

alpha-Acetolactate Decarboxylase; Origin: Bacillus subtilis
Fluka — Lab

Lysine decarboxylase. **4.1.1.18**

L-lysine = cadaverine + CO(2).

L-Lysine Decarboxylase; Origin: Bacterium cadaveris
Fluka — Lab

Tyrosine decarboxylase. **4.1.1.25**

L-tyrosine = tyramine + CO(2).

L-Tyrosine Decarboxylase; Origin: Streptococcus faecalis
Fluka — Lab

Phosphoenolpyruvate carboxylase. **4.1.1.31**

Phosphate + oxaloacetate = H(2)O + phosphoenolpyruvate + CO(2).

Phosphoenolpyruvate carboxylase; Origin: maize leaves
Biozyme — Pilot

Phosphoenolpyruvate Carboxylase; Origin: maize leaves
Fluka — Lab

Phosphoenolpyruvate Carboxylase; Origin: microorganisms
Toyobo — Pilot

Phenylalanine decarboxylase. **4.1.1.53**

L-phenylalanine = phenethylamine + CO(2).

L-Phenylalanine Decarboxylase; Origin: Streptococcus faecalis
Fluka — Lab

Methionine decarboxylase. **4.1.1.57**

L-methionine = 3-methylthiopropanamine + CO(2).

L-Methionine decarboxylase; Origin: Streptomyces sp.
Fluka — Lab

Deoxyribose-phosphate aldolase. **4.1.2.4**

Phosphodeoxyriboaldolase. Deoxyriboaldolase.

2-deoxy-D-ribose 5-phosphate = D-glyceraldehyde 3-phosphate +acetaldehyde.

2-Deoxyribose-5-phosphate aldolase; Origin: Lactobacillus plantarum
Fluka — Lab

Threonine aldolase. **4.1.2.5**

L-threonine = glycine + acetaldehyde.

Threonin Aldolase ; Origin: Candida humicola
Fluka — Lab

Threonin Aldolase; Origin: Pseudomonas putida
Fluka — Lab

Mandelonitrile lyase. **4.1.2.10**

Hydroxynitrile lyase. (R)-oxynitrilase.

Mandelonitrile = cyanide + benzaldehyde.

(R)-Oxynitrilase; Origin: bitter almonds (Prunus amygdalus)
Jülich Enzyme Products — Lab

Table 20.5. (cont.).

R-Oxynitrilase rec.; Origin: Pichia pastoris
Fluka — Pilot

Hydroxymandelonitrile lyase. — **4.1.2.11**
Hydroxynitrile lyase. — (S)-4-hydroxymandelonitrile = cyanide + 4-hydroxybenzaldehyde.

(S)-Oxynitrilase; Origin: Sorghum bicolor or S. vulgare
Jülich Enzyme Products — Lab

Fructose-bisphosphate aldolase. — **4.1.2.13**
Aldolase. Fructose-1,6-bisphosphate triosephosphate-lyase. — D-fructose 1,6-bisphosphate = glycerone phosphate + D-glyceraldehyde 3-phosphate.

Fructose-1,6-bisphosphate aldolase; Origin: Bacillus subtilis
Fluka — Lab

Aldolase; Origin: rabbit muscle
Fluka — Lab

Aldolase; Origin: rabbit muscle
Roche Diagnostics: Aldolase — Lab

Aldolase; Origin: Staphylococcus aureus
Fluka — Lab

Aldolase ; Origin: Staphylococcus carnosus
Fluka — Lab

Fructose 1,6-bisphosphate Aldolase; Origin: Staphylococcus carnosus
Jülich Enzyme Products — Lab

Aldolase; Origin: Thermus aquaticus
Fluka — Lab

2-dehydro-3-deoxyphosphogalactonate aldolase. — **4.1.2.21**
6-phospho-2-dehydro-3-deoxygalactonate aldolase.
6-phospho-2-keto-3-deoxygalactonate aldolase.
2-oxo-3-deoxygalactonate 6-phosphate aldolase. — 2-dehydro-3-deoxy-D-galactonate 6-phosphate = pyruvate +D-glyceraldehyde 3-phosphate.

6-Phospho-2-dehydro-3-deoxygalactonate aldolase
Fluka — Lab

Dihydroneopterin aldolase. — **4.1.2.25**
2-amino-4-hydroxy-6-(D-erythro-1,2,3-trihydroxypropyl)-7,8-dihydr opteridine = 2-amino-4-hydroxy-6-hydroxymethyl-7,8-dihydropteridine +

Dihydroneopterin Aldolase
Fluka — Lab

Hydroxynitrilase. — **4.1.2.39**
Hydroxynitrile lyase. Oxynitrilase. — 2-hydroxyisobutyronitrile = cyanide + acetone.

Hydroxynitrile Lyase; Origin: Hevea brasiliensis, rec. Pichia sp.
Roche Diagnostics: Hydroxynitrile Lyase (HNL) — Industrial

N-acetylneuraminate lyase. — **4.1.3.3**
N-acetylneuraminic acid aldolase. — N-acetylneuraminate = N-acetyl-D-mannosamine + pyruvate.

N-Acetyl-neuraminic acid aldolase; Origin: E. coli
Fluka — Lab

Neuraminic Acid Aldolase; Origin: E. coli K12
Jülich Enzyme Products — Lab

N-Acetylneuraminic Acid Aldolase; Origin: microorganisms
Toyobo — Pilot

Table 20.5. (cont.).

N-Acetylneuraminic Acid Aldolase; Origin: microorganisms
Unitika: N-Acetylneuraminic Acid Aldolase (Nana-Ald) — Pilot

Citrate lyase. — **4.1.3.6**
Citrase. Citratase. Citritase. Citridesmolase.
Citrate = acetate + oxaloacetate.

Citrate Lyase; Origin: Enterobacter aerogenes
Fluka — Lab

4-hydroxy-2-oxoglutarate aldolase. — **4.1.3.16**
2-keto-4-hydroxyglutarate aldolase. 2-oxo-4-hydroxyglutarate aldolase. KHG-aldolase.
4-hydroxy-2-oxoglutarate = pyruvate + glyoxylate.

4-Hydroxy-2-oxoglutarate aldolase ; Origin: E.coli
Fluka — Lab

4-hydroxy-4-methyl-2-oxoglutarate aldolase. — **4.1.3.17**
4-hydroxy-4-methyl-2-oxoglutarate = 2 pyruvate.

4-Hydroxy-4-methyl-2-oxoglutarate aldolase
Fluka — Lab

Tryptophanase. — **4.1.99.1**
L-tryptophan indole-lyase. Tnase.
L-tryptophan + H(2)O = indole + pyruvate + NH(3).

Tryptophanase; Origin: microorganisms
Unitika: Tryptophanase (Trp) — Pilot

Tyrosine phenol-lyase. — **4.1.99.2**
Beta-tyrosinase.
L-tyrosine + H(2)O = phenol + pyruvate + NH(3).

beta-Tyrosinase; Origin: microorganisms
Unitika: beta-Tyrosinase (Bty) — Pilot

Carbonate dehydratase. — **4.2.1.1**
Carbonic dehydratase. Carbonic anhydrase.
H(2)CO(3) = CO(2) + H(2)O.

Carbonic anhydrase; Origin: Bbvine erythocytes
Biozyme — Pilot

Carbonic Anhydrase; Origin: bovine erythrocytes
Fluka — Lab

Carbonic Anhydrase Isozyme II; Origin: bovine erythrocytes
Fluka — Lab

Chondroitin ABC lyase. — **4.2.2.4**
Chondroitinase. Chondroitin ABC eliminase.
Eliminative degradation of polysaccharides containing 1,4-beta-D-hexosaminyl and 1,3-beta-D-glucuronosyl or 1,3-alpha-L-iduronosyl linkages to disaccharides containing

Chondroitinase ABC; Origin: Proteus vulgaris
Fluka — Lab

Pectin lyase. — **4.2.2.10**
Eliminative cleavage of pectin to give oligosaccharides with terminal 4-deoxy-6-methyl-alpha-D-galact-4-enuronosyl groups.

Pectolyase (EC 3.2.1.15 & 4.2.2.10); Origin: Aspergillus japonicus
Fluka — Industrial

Phenylalanine ammonia-lyase. — **4.3.1.5**
L-phenylalanine = trans-cinnamate + NH(3).

Phenylalanine Deaminase; Origin: Rhodotorula glutinis
Fluka — Lab

Table 20.5. (cont.).

L-3-cyanoalanine synthase. **4.4.1.9**

L-cysteine + cyanide = H(2)S + L-3-cyanoalanine.

beta-Cyanoalanine Synthase; Origin: microorganisms
Unitika: beta-Cyanoalanine Synthase (Bcs) — Pilot

Alanine racemase. **5.1.1.1**

L-alanine = D-alanine.

Alanine Racemase; Origin: Bacillus stearothermophilus
Unitika: Alanine Racemase (AlaR) — Pilot

Aldose 1-epimerase. **5.1.3.3**

Mutarotase. Aldose mutarotase.

Alpha-D-glucose = beta-D-glucose.

Mutarotase; Origin: hog kidney
Amano: Amano 2 [MUT-2] — Pilot

Mutarotase; Origin: pig kidney
Biozyme — Pilot

Triosephosphate isomerase. **5.3.1.1**

Triosephosphate mutase. Phosphotriose isomerase.

D-glyceraldehyde 3-phosphate = glycerone phosphate.

Triosephosphate isomerase; Origin: rabbit muscle
Biozyme — Pilot

Xylose isomerase. **5.3.1.5**

D-xylose = D-xylulose.

Glucose isomerase; Origin: microorganisms
Novozymes: Sweetzyme® — Industrial

Glucose-6-phosphate isomerase. **5.3.1.9**

Phosphoglucose isomerase. Phosphohexose isomerase. Phosphohexomutase. Oxoisomerase.

D-glucose 6-phosphate = D-fructose 6-phosphate.

Phosphoglucose Isomerase; Origin: Bacillus stearothermophilus
Unitika: Phosphoglucose Isomerase (PGI) — Pilot

Phosphoglucose Isomerase; Origin: baker's yeast
Fluka — Pilot

Protein disulfide isomerase. **5.3.4.1**

S-S rearrangase.

Rearrangement of both intrachain and interchain disulfide bonds inproteins to form the native structures.

Protein disulfide Isomerase; Origin: bovine liver
Fluka — Lab

Protein disulfide Isomerase; Origin: E. coli
Fluka — Lab

Phosphoglucomutase. **5.4.2.2**

Glucose phosphomutase. Phosphoglucose mutase.

Alpha-D-glucose 1-phosphate = alpha-D-glucose 6-phosphate.

Phosphoglucomutase; Origin: rabbit muscle
Fluka — Lab

Long-chain-fatty-acid--CoA ligase. **6.2.1.3**

Acyl-activating enzyme. Acyl-CoA synthetase. Fatty acid thiokinase (long-chain). Lignoceroyl-CoA synthase.

ATP + a long-chain carboxylic acid + CoA = AMP + diphosphate + anacyl-CoA.

Acyl-CoA Synthetase; Origin: microorganisms
Asahi — Pilot

Table 20.5. (cont.).

Acyl-CoA Synthetase; Origin: Pseudomonas sp.
Amano: Amano 2 [ACS-2] — Pilot

Acyl-CoA Synthetase; Origin: Pseudomonas sp.
Amano: Amano 3 [ACS-3] — Pilot

Acyl-coenzyme A Synthetase; Origin: Pseudomonas sp.
Fluka — Lab

Glutamate--ammonia ligase. **6.3.1.2**
Glutamine synthetase. ATP + L-glutamate + NH(3) = ADP + phosphate + L-glutamine.

Glutamine Synthetase; Origin: Bacillus stearothermophilus
Unitika: Glutamine Synthetase (GS) — Lab

NAD(+) synthase. **6.3.1.5**
NAD(+) synthetase. ATP + deamido-NAD(+) + NH(3) = AMP + diphosphate + NAD(+).

NAD Synthetase
Asahi — Pilot

Urea carboxylase. **6.3.4.6**
Urease (ATP-hydrolysing). Urea carboxylase (hydrolysing). ATP--urea amidolyase. Urea amido-lyase. ATP + urea + CO(2) = ADP + phosphate + urea-1-carboxylate.

Urea Amidolyase; Origin: yeast
Toyobo — Pilot

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